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STUDIES IN CANNING PROCESSES
III. THE COOLING PHASE OF PROCESSES FOR PRODUCTS HEATING BY CONDUCTION

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(Manuscript received August 17, 1959)

In canned foods which heat by conduction, the cooling phase of the retort process makes a substantial contribution to the destruction of bacterial spores and to the lethality (F value) of the process. It is important therefore, to evaluate the contribution of the cooling phase (F_c) as accurately as possible when evaluating the lethality of a process.

Three methods for calculating F_c have been proposed. In the general method of process evaluation of Bigelow, Bohart, Richardson and Ball *(3)*, and the variants of it *(11, 13)* the calculations are based on experimentally determined temperature histories of test cans. In Ball's formula method *(1)* and other methods based on it *(10)* a standard form of cooling curve is assumed and calculations are based on this curve. Ball's cooling curve is of a simple mathematical form which was selected to represent extensive experimental data; it differs from curves obtained when cooling is purely by conduction and represents faster cooling. Gillespy's method *(6)* for evaluating the contribution of the cooling phase is based on the assumption that cooling in conduction heating packs is purely by conduction. Gillespy worked out a convenient approximate method which simplifies the otherwise cumbersome mathematics associated with conduction cooling.

Under certain conditions packs which heat by conduction may cool purely by conduction *(7, 8)* but under other conditions there may be movement of the product in the can causing faster cooling *(7, 12)*. The effect on F_c of the change from one cooling mechanism to the other may be large. For instance, Hemler, Alstrand, Ecklund and Benjamin *(7)* working with 603 x 700 cans of cream style corn processed at 250° F reported an F value of 4.8 min when the pressure in the retort was held at 20 psig during cooling and 1.8 min when the pressure was quickly reduced. The latter value agreed closely with that calculated by Ball's method.

Because Ball's method is based on a standard form of the cooling curve which is substantially different from curves obtained experimentally and because Gillespy's method depends on the product cooling purely by conduction, it seemed appropriate to obtain further data to assess the agreement of the two mathematical methods with the general method of Bigelow *et al.*

EXPERIMENTAL PROCEDURES

The experimental work involved measuring the temperature at the slowest heating point *(5)* in cans of foods heating by conduction during the heating and cooling phases of retort processes.

* Deceased, November 2, 1959.

Temperature measurements. Can temperatures and the temperature of the steam and cooling water adjacent to the cans were measured during heating and cooling using 34 S.W.G. nichrome-constantan thermocouples. The cotton covered and lacquered wires entered the cans through diametrically opposite holes in the cylindrical wall. The wires were sealed in the holes in the can wall with a heat resistant epoxy resin. The hot junction was formed by soldering the wires together and the insulation of the wires at the junction was repaired by lacquering. This technique fixed the position of the hot junction without introducing materials other than the wires into the can. Under these conditions errors in the measurement of can temperatures caused by conduction of heat along the wires were insignificant *(4)*. Except at times when the temperature changed rapidly measurements were made to an accuracy of $\pm 0.1°$ F.

Retorts. Temperature measurements were made in a small laboratory retort and in two different retorts in commercial canneries.

The laboratory retort was used to process 4 replicate cans under carefully controlled conditions to provide information on the effect of different methods of retort operation on the cooling of different packs. The temperature of the laboratory retort was controlled at about 240° F or 250° F to $\pm$ 1° F during the heating phase. The replicate cans in each treatment were placed in a special container in the retort so that cooling water sprayed on the cans immediately after water entered the retort, and covered the cans completely 30 sec after that time. Separate streams of water circulated round each can and then ran to waste so that the water temperature remained practically constant throughout cooling. The retort was fitted with a manually controlled compressed air supply which was used to purge the retort of steam at the beginning of cooling and to maintain pressure in the retort during cooling. Purging the retort of steam required 15–20 sec and adjusting the pressure required about 10 sec. The air pressure was controlled to $\pm$ 1 psi during cooling until the center temperature in the slowest cooling can reached 212° F; the retort pressure was then quickly reduced to atmospheric pressure. Water cooling continued until all cans reached 190° F.

The measurements in the industrial retorts were designed to give information on the variation in the cooling rate of cans in different parts of the retorts. Most of the measurements were made in a vertical retort which had the cooling water inlet at the bottom and the outlet near the top. The other measurements were made in a horizontal retort which had the water inlet at the bottom and the outlet in the top of the retort vertically above the inlet. In contrast to the laboratory retort which contained only thermocouple cans during the measurements, the industrial retorts were fully loaded with cans from normal production and the cooling water was turned on immediately after the steam was turned off.

Test packs. The test packs used in the laboratory retort were baked beans, cream style corn, tomato puree (9% soluble solids) and meat loaf in 401 x 411 cans; baked beans and tomato puree in 301 x 411 cans and solid meat in 301 x 308 cans. These packs were used in 39 heat penetration runs covering different levels of retort temperature, can vacuum, retort pressure during cooling, delay between "steam off" and "water on," and g (the difference between the retort temperature and can center temperature at "steam off").

The test packs in the industrial retorts were vegetable soup in 301 x 411 and 301 x 211 cans, spaghetti bolognaise in 301 x 411 cans, baked beans in 603 x 700 cans and curried chicken in 401 x 211 cans.

Treatment of temperature data. The heat penetration data obtained from each can were treated in the following way.

1. The can temperature was plotted against time for the heating and cooling phases.
2. The logarithm of the difference between the retort temperature and the can temperature was plotted against time for the heating phase. An asymptote was fitted to the straight line part of the curve by eye and the slope of the asymptote (f_h) was measured.
3. The lethality of the heating phase (F_h) was calculated by the method of Bigelow *et al* and by the formula method using the equation given by Ball and Olsen (*2* Eq 12.52) and Tables on Exponential Integrals *(9)*. The heating phase was considered to end when the steam was turned off.

4. For the measurements in the laboratory retort the lethality of the delay period (F_d), the period between "steam off" and "water on," was calculated by the graphical method and by Ball's equation for the heating phase assuming the delay period to be a simple extension of the heating phase.
5. The cooling phase for measurements in the laboratory retort was considered to start at "water on" and the lethality (F_c) of this part of the process was calculated by the general method and the formula methods of Ball and of Gillespy. Ball's Table of his ρ function (*2* Table 12.4 p. 346) was used for calculation by this method.
6. In the industrial retorts there were delays of up to 7 min from the time of "steam off—water on" until the last of the test cans were immersed in cooling water. It was considered that in the practical application of Ball's and Gillespy's methods for calculating F_c it must be assumed that the cans were immersed in water at "steam off." Consequently F_c was calculated by the general method, by Ball's method and by Gilespy's method on the assumption that all test cans were immersed in water at "steam off." In addition to the variations in delay time in different parts of the industrial retorts, the cooling water temperature in the neighborhood of particular cans varied as cooling progressed. For the purpose of calculating F_c by Ball's and Gillespy's methods the tempeature of the cooling water was assumed to be constant and equal to the temperature of the water entering the retort.

RESULTS

Results of the measurements in the laboratory retort and calculations on the temperature data are given in Table 1. The data in this table are means of 4 replicate cans.

Results of the measurements on the cans in the industrial retorts and the position of the test cans in the retorts are recorded in Table *2*; the data in this table are for single cans.

DISCUSSION

Evaluation of the heating phase. It is evident from Tables 1 and 2 that F_h values calculated by the general method and by the formula method agreed closely; the mean ratio of F_h calculated by the formula method to that calculated by the graphical method for the data in Table 1 is 1.0008 ± 0.0019. This ratio is clearly not significantly different from unity.

Discrepancies between the two estimates of F_h may arise from errors of calculation, particularly in fitting the asymptote to a heating curve and determining its slope and in performing the graphical integrations. There may also be deviations due to curvature or irregularities in the later parts of the heating curve. The individual ratios of F_h calculated by the two methods, were not normally distributed. Approximately 75% of the ratios differed from unity by less than 1% and these discrepancies can probably be attributed to errors of calculation. The maximum deviation was 3.2% and 10% of the discrepancies exceeded 2%; in all these cases the semi-logarithmic plots of the experimental heating data showed curvature or irregularities. Thus these larger discrepancies are probably due largely to experimental errors rather than errors of calculation.

Evaluation of the cooling phase.

a. Measurements in the laboratory retort. The cooling curves obtained from most of the experimental treatments in which the retort pressure was maintained at 15 psig were similar to cooling curves calculated mathematically for purely conduction cooling. The cooling curves obtained when the retort pressure was reduced to 5 psig or less during cooling showed faster cooling than that calculated for the conduction mechanism and some of these experimental curves showed irregular fluctuations in rate of cooling. It appears

TABLE 1

Lethality of retort processes at approximately 240° F given to conduction heating canned food in laboratory retort

Treatment No.	Can vacuum *(in. Hg)*	Retort air pressure *(psig)*	Delay *(min)*	g (° F)	Heating phase (F_h)		Delay phase (F_d)		Cooling phase (F_c)		
					General method	Formula method	General method	Formula method	General method	Ball's method	Gillespy's method
Baked beans in No. 2½ (401 x 411) cans											
1	11.8	15	1	3.08	6.29	6.43	0.17	0.14	3.02	1.22	2.88
2	14.6	15	10	3.25	5.73	5.71	1.67	1.69	2.89	1.24	2.89
3	12.2	5	1	2.90	7.43	7.36	0.19	0.22	2.08	1.37	3.21
4	16.9	5	10	2.68	8.21	8.14	2.05	2.12	1.54	1.53	3.63
5	5.5	15	1	3.44	7.27	7.38	0.19	0.21	3.54	1.51	3.56
6	5.2	15	10	3.23	6.86	6.87	1.93	1.93	3.16	1.50	3.48
7	3.6	5	1	3.44	6.49	6.52	0.18	0.18	1.72	1.35	3.16
8	4.7	5	10	2.90	7.54	7.59	2.00	2.05	1.61	1.57	3.62
9	15.3	15	1	9.51	1.45	1.46	0.08	0.08	1.79	0.55	1.61
10	16.0	15	10	9.13	1.60	1.62	0.94	0.96	1.83	0.78	2.05
11	13.0	5	1	9.25	1.69	1.70	0.09	0.09	1.45	0.63	1.83
12	15.2	5	10	9.36	1.77	1.77	0.09	1.10	1.38	0.86	2.31
13	5.1	15	1	9.29	1.80	1.81	0.09	0.10	2.00	0.68	1.97
14	5.9	15	10	9.65	1.66	1.68	1.07	1.04	2.18	0.83	2.26
15	5.6	5	1	9.22	2.01	2.04	0.10	0.12	1.26	0.74	2.17
16	4.6	5	10	9.74	1.52	1.53	0.92	0.98	1.35	0.79	2.11
17	4.8	0	0	3.37	3.64	3.63	.....	.....	1.40	0.72	1.57
18	14.7	0	0	2.96	4.69	4.64	.....	.....	1.05	0.85	1.85
19	6.0	0	0	9.05	1.08	1.09	.....	.....	0.87	0.40	1.03
20	17.1	0	0	9.54	0.87	0.87	.....	.....	0.69	0.32	0.86
20[1]	17.0	0	0	9.19	0.96	0.97	.....	.....	0.76	0.36	0.94
21	5.3	0	0	2.87	7.46	7.23	.....	.....	1.96	1.33	3.10
21[1]	5.2	0	0	2.70	8.39	8.45	.....	.....	2.19	1.50	3.50
22	11.7	0	0	3.10	6.39	6.41	.....	.....	1.51	1.22	2.88
23	6.4	0	0	9.80	1.48	1.49	.....	.....	1.02	0.57	1.66
24	14.4	0	0	8.73	1.91	1.90	.....	.....	0.67	0.67	1.90
25[2]	9.0	0	0	9.51	5.04	5.08	.....	.....	2.75	1.87	5.42

TABLE 1 (continued)

Treatment No.	Can vacuum (*in. Hg*)	Retort air pressure (*psig*)	Delay (*min*)	g (° F)	Heating phase (F_h)		Delay phase (F_d)		Cooling phase (F_c)		
					General method	Formula method	General method	Formula method	General method	Ball's method	Gillespy's method
Cream style corn No. 2½ (401 x 411) cans											
26	10.1	15	1	9.61	1.59	1.62	0.09	0.10	1.89	0.62	1.79
27	9.9	5	1	9.79	1.46	1.46	0.09	0.08	1.50	0.56	1.66
28	1.5	15	1	9.75	1.59	1.61	0.09	0.09	1.88	0.61	1.83
29	2.7	5	1	9.34	1.75	1.75	0.09	0.09	1.50	0.64	1.89
Meat loaf in No. 2½ (401 x 411) cans											
30	5.4	15	1	9.83	1.45	1.40	0.07	0.06	1.46	0.51	1.54
31	6.8	5	1	9.93	1.25	1.25	0.06	0.06	1.09	0.48	1.41
Pre-shrunk meat in (301 x 308) cans											
32	8.8	5	1	9.63	0.88	0.86	0.08	0.08	0.51	0.36	1.00
33	10.8	15	1	9.62	0.90	0.90	0.08	0.09	0.62	0.38	1.04
Tomato pulp in No. 2½ (401 x 411) cans											
34	8.0	5	1	8.58	1.61	1.60	0.08	0.09	1.11	0.60	1.63
35	7.3	15	1	8.92	1.58	1.59	0.08	0.08	0.91	0.62	1.69
36	10.8	0	0	8.00	1.85	1.85	.....	.....	0.48	0.63	1.71
Tomato pulp in 1 lb (301 x 411) cans											
37	11.2	0	0	1.13	8.57	8.46	.....	.....	0.76	1.01	2.14

[1] Repetition of preceding treatment.
[2] Retort temperature 250° F.

TABLE 2

Lethality of processes given to conduction heating foods situated in different positions in industrial retorts

Can No.	Position of can in retort	Heating phase (F_h)		Cooling phase (F_c)		
		General method	Formula method	General method	Ball's method	Gillespy's method
Spaghetti Bolognaise in 1 lb (301 x 411) cans processed 60 min at 250° F in vertical retort						
1	Bottom of retort near water inlet	1.97	1.93	1.47	0.63	2.29
2	Top of retort opposite water outlet	1.86	1.85	1.99	0.88	2.37
3	Centre top crate of cans	2.16	2.17	1.85	0.93	2.67
4	Same level as 3 but against crate wall	1.78	1.78	1.68	0.79	2.25
Baked beans in No. 10 (603 x 700) cans processed 240 min at 240° F in vertical retort						
5	Bottom of retort near water inlet	3.38	3.45	1.50	1.20	4.02
6	Top of retort opposite water outlet	4.08	4.11	1.50	1.41	4.48
7	Centre top crate of cans	4.40	4.41	1.52	1.46	4.59
8	Same level as 7 but against crate wall	4.39	4.42	1.41	1.44	4.62
Vegetable soup in 1 lb (301 x 411) cans processed 45 min at 240° F in vertical retort						
9	Bottom of retort near water inlet	0.192	0.191	0.323	0.106	0.305
10	Top of retort opposite water outlet	0.169	0.172	0.531	0.111	0.285
11	Centre top crate of cans	0.216	0.218	0.470	0.129	0.351
12	Same level as 11 but against crate wall	0.180	0.180	0.399	0.101	0.290
Vegetable soup in 8 oz (301 x 209) cans processed 45 min at 240° F in vertical retort						
13	Bottom of retort near water inlet	0.612	0.611	0.622	0.237	0.757
14	Top of retort opposite water outlet	0.890	0.891	1.406	0.343	0.939
15	Centre top crate of cans	0.783	0.780	0.961	0.316	0.920
16	Same level as 15 but against crate wall	0.727	0.737	0.862	0.290	0.891
Curried chicken in 1 lb (401 x 211) cans processed 95 min at 239° F in horizontal retort						
17	Bottom of retort near water inlet	3.82	3.85	0.47	0.77	1.66
18	Top of retort away from water outlet	4.12	4.08	1.13	0.81	1.74
19	Centre of crate of cans	3.80	3.81	0.78	0.76	1.66
20	Same level as 19 but against crate wall	3.78	3.92	0.83	0.76	1.64

therefore that the mechanism of cooling depended on the way in which cooling was carried out; under some conditions cooling was predominately by conduction while in other cases it was accelerated by movement of the content of the can. Although most of the cans which were cooled under 15 psig superimposed pressure did so by conduction, others, such as those in Treatments No. 33 and 35 in Table 1, cooled faster. Examples of the cooling curves obtained experimentally when cooling was predominately by conduction and when cooling was accompanied by movement of the contents of the can are shown in Figure 1. In addition a cooling curve calculated for purely conduction cooling and one calculated from Ball's equations (*2*. Cr. 12) are also shown in Figure 1.

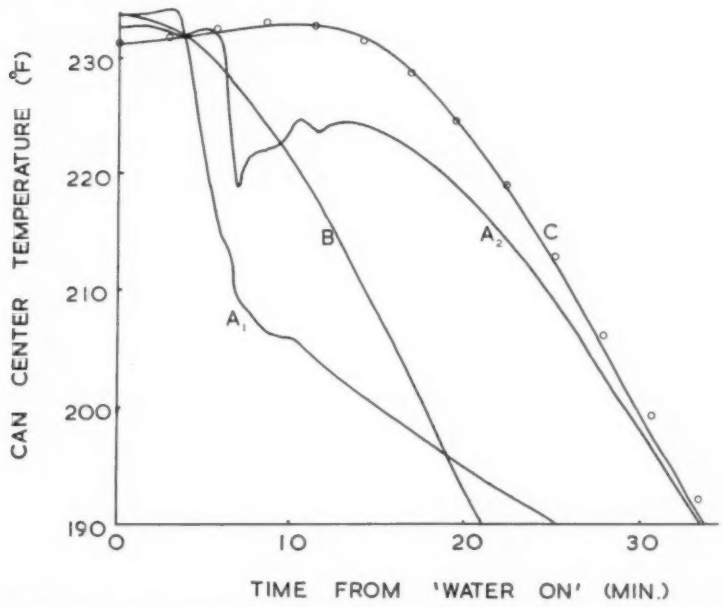

Figure 1. Cooling curves for 401x411 cans of baked beans. Curves A_1 and A_2 represent extreme examples of irregular cooling with 5 psig retort pressure. Curve B was calculated from Ball's equation. Curve C is an experimental cooling curve with 15 psig. retort pressure. The points plotted are calculated values for cooling by conduction, assuming the f_h and g values applying to curve C.

In the more viscous products such as baked beans, cream-style corn and solid meat, boiling with consequent movement of material in the can is probably the main cause of non-conductive cooling. Convective processes may contribute to this effect especially in the cast of tomato pulp. In the 401 x 411 cans which cooled by the non-conductive process it was possible to show by calculation that the temperature and pressure conditions in the cans at the moment the cans were immersed in the cooling water were such that boiling would take place during the early part of the cooling

phase. The calculations also showed that F_c decreased as the pressure in the can decreased. These calculations involved such variables as the pressure-volume relationship of the cans and were subject to large can-to-can variations. It is therefore considered they have no practical application in the calculation of F_c and are reported here only to support the hypothesis that movement in the can is at least partly caused by boiling.

In the cans which cooled predominately by conduction, F_c calculated by Gillespy's method which assumes conduction cooling, agreed satisfactorily with F_c calculated graphically. Examples of this agreement are shown in Treatments Nos. 1, 2, 5 and 6 in Table 1. In cans cooling with movement of the product F_c calculated by Gillespy's method was larger by factors up to 2.4 than F_c calculated by the graphical method (e.g. Treatments Nos. 3, 4, 7 and 8 in Table 1). In some treatments the overestimation of F_c by Gillespy's method was large enough to increase seriously the risk of underprocessing.

F_c calculated by Ball's method was less than F_c calculated graphically except in Treatments Nos. 4, 8 and 24 where the two methods gave approximately the same results and in Treatment No. 36 where Ball's F_c was greater than F_c calculated graphically. In this last case the discrepancy was not large and it occurred in a product which was unusual in that it heated by conduction and cooled mainly by convection.

It seems likely that in determining the cooling curves on which his method is based, Ball used thermocouples which were subject to conduction errors *(4)* which would result in faster cooling and the underestimation of F_c.

It should be noted that neither Ball's nor Gillespy's methods take account of the contribution of the delay period in estimating F_c. The values of F_d in Table 1 calculated by the formula method involve the assumption that the lag period was equivalent to a simple extension of the heating phase. These estimates agree well with values derived by the general method which involves no simplifying assumptions. The theoretical grounds for expecting such agreement are that the flux of heat at the surface is small in the later stages of heating and the decrease in this flux during the lag period will also be small. Furthermore, the effect at the center of a change in flux at the surface will only become apparent after a delay of several minutes. Thus any error introduced by the simplifying assumption might affect F_c more than F_d.

An approximate theoretical estimate of this error was obtained from solutions of the relevant equations for conduction in an infinite cylinder, which are much more convenient for calculation than those for a finite cylinder. Considering an infinite cylinder 10 cm in diameter, of thermal diffusivity 0.0866 cm.^{2}/min, assuming $g = 10°$ F, retort temperature 240° F, cooling water temperature 60° F, and a delay of 10 min during which there was no exchange of heat between the can and its surroundings, the calculated error in F_d introduced by treating the lag period as an extension of the heating phase was negligible and the error in F_c approximately 1%. This is clearly small compared with the variations in F_c due to other causes.

b. Measurements in the industrial retorts. The data in Table 2 show that the value of F_c calculated by the general method varied with position

in the retort. The value of F_c was least in cans which were immersed in cold water immediately after the water was turned on. Higher values were found elsewhere, presumably because of the delay between "water on" and the actual immersion of the cans, and warming of the water by cans nearer the water inlet.

The magnitude of the effect of position in the retort varied from one series of measurements to another. The greatest effect was found in the 401 x 211 squat cans of curried chicken processed in a horizontal retort. In this case F_c measured in a can near the water inlet was about 40% of F_c measured in a can near the top of the retort and away from the water outlet. The measurements in the vertical retort indicate that the variation of F_c with position in the retort increased as the can size decreased, probably because warming of the water in the early stages was greater in small cans with a high surface to volume ratio.

F_c values calculated by Ball's method in most cases were less than F_c calculated graphically. The greatest discrepancy occurred in one of the 301 x 411 cans of vegetable soup when the graphical F_c was 4.8 times that calculated by Ball's method (Can 10, Table 2).

F_c values calculated by Gillespy's method were as much as 3 times larger than F_c determined graphically but in other cans Gillespy's F_c was less than the graphical F_c. As in the case of Ball's method, the largest underestimation of F_c again occurred in Can 10 in which Gillespy's method gave a value for F_c about half that of F_c calculated graphically. The experimental records show that the supply of cooling water to this can was unusually poor. The temperature measured beside the can was 143° F 5 min after water was turned on and 108° F after 10 min.

It is evident that the value of F_c in industrial retorts is inherently a very variable quantity. It varies with the position in the retort, the cooling schedule, the nature of the product, the size of the can and other factors. In evaluating processes it is probably wise therefore to consider only the fastest cooling cans in the retort. F_c for these cans, which are usually near the water inlet, may vary between any upper limit corresponding to cooling purely by conduction, which may be calculated by Gillespy's method, and a lower limit which is ill-defined but which seems to be generally close to the estimate obtained by Ball's method. The difference between these two limiting values can be quite large, e.g. about 3 min equivalent time at 250° F in commercial processes for baked beans in 603 x 700 cans.

c. Practical considerations. The measurements in the laboratory retort show that controlled pressure cooling, particularly when the can vacuum is not high, or the use of a delay period increased F_c or $(F_d + F_c)$ and consequently would allow some reduction in the severity of the heating process. Increasing F_c by means of controlled pressure cooling may be of commercial importance in the processing of products whose quality is impaired by heating for the times usually recommended. However the value of using a delay period to reduce heat damage may be limited. Any industrial attempt to make use of the possibility of increasing F_c above the value commonly assumed and reduce F_h accordingly would need to be very carefully controlled and tested.

It seems most improbable that any useful mathematical method could be developed for calculating F_c in cases where it exceeds the Ball estimate

(unless cooling purely by conduction could be assumed). Consequently experimental determination of F_c by the general method would be necessary. Fine wire thermocouples should be used for the temperature measurements in any critical study of F_c. It would be best to work in the retorts in which the process is to be applied. If laboratory retorts were used for such tests particular care would be needed to reproduce the time, pressure and temperature schedules of the industrial retorts.

SUMMARY

The contribution of the cooling phase to the lethal value of a process (F_c) varied widely with the nature of the pack and the processing conditions.

If a can was immersed in cooling water immediately steam was cut off, the value of F_c varied between an upper limit given by Gillespy's method of calculation and a lower limit which was ill-defined but seemed generally to be close to the value calculated by Ball's formula.

In all but one of the experimental runs, Ball's method underestimated F_c, often by a factor exceeding 2. F_c determined by the general method was less than Ball's estimate in only one of the twenty cans measured in the industrial retort; in this can the discrepancy was 0.3 min.

Maintenance of an air pressure of 15 psig in the retort during cooling tended to increase F_c particularly when the can vacuum was not high. Values of F_c close to the estimate given by Gillespy's method were often obtained with careful pressure cooling.

Delay between cutting off steam and immersing the cans in cold water increased the lethal value of a process substantially. This was a major source of variation in F_c in industrial retorts.

There seem to be possibilities of improving the quality of some sensitive products by careful control of cooling conditions to ensure a high F_c value and reducing the length of the heating phase correspondingly.

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RHEOLOGY OF COCOA BUTTER. I. EFFECT OF CONTAINED FAT CRYSTALS ON FLOW PROPERTIES

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Rheological properties of molten chocolate have been the subject of extensive studies *(1, 3, 4, 6, 7, 14)*. Basically, the aims of these studies have been twofold: (1) to characterize the so-called covering power of a particular chocolate formulation; and (2) to estimate the quality of the congealed chocolate in terms of fineness of crystal, "snap," etc. Investigations of flow properties are generally carried out at a temperature above the melting point of the most stable (β) crystal form of the cocoa butter—usually 100° F (37.8° C)—so that the fatty component of the chocolate will be entirely fluid. Nevertheless, numerous suspended particles (sugar, milk protein, vegetable cell wall components, starch grains, etc.) will be present. These necessarily cause a marked non-Newtonian behavior of the chocolate mass.

Many of the quality factors which are to be related to rheological characteristics depend upon the nature of crystallization of the cocoa butter. Hence, it appears that their estimation by means of physical properties of fully molten chocolate is rather indirect. From an *a priori* standpoint, a more obvious method of analyzing the crystallizability of the fats of a chocolate would be to study the effect of crystallization in cocoa butter on the viscous properties of the latter. It is well known that suspended particles will influence the flow behavior of a fluid *(9, 10, 12, 13, 15)*. According to the Einstein equation for spheres in dilute solution, the relationship is of the following nature: $\eta_{sp} = 1 + 2.5\phi$, where η_{sp} is the specific viscosity and ϕ is the volume concentration of the spheres. Hence, if small fat crystals are treated as spheres, it could be possible to determine, by viscous properties alone, their relative concentration. In concentrated solutions, further terms appear on the right hand side of the equation, proportional to higher powers of ϕ, and the viscous effect is more pronounced.

It should be noted, however, that the size of the crystalline particle, an important element in cocoa butter quality, cannot be derived independently from viscosity measurements. Nevertheless, it was considered useful to explore the relationship of viscous properties with crystallinity. Such an investigation might be more meaningful than a study of flow properties of the non-crystalline cocoa butter or of the molten chocolate mass. Also, it seemed reasonable to expect that incipient crystallinity might be detected by viscous changes, perhaps before the development of microscopically visible crystals.

MATERIALS AND METHODS

An Ubbelohde type of viscometer was constructed, consisting of a horizontal capillary tube (0.5 mm diameter bore and 30 cm length) between two vertical cylindrical reservoirs of equal height, shape, and volume. The capillary tube connected the reservoirs at their bases. Transition between the capillary bore and the reservoir lumen was continuous, through a 90° turn.

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Each reservoir was connected to a two-way stopcock. The latter gave access either to the atmosphere or to the pressure system which was used to induce flow. Flow could occur only when one reservoir was under applied pressure and the other open to the atmosphere. The pressure system consisted of a compressed air source, a 40-liter storage vessel, and a water or mercury manometer, open to the atmosphere on one side and connected in parallel to the viscometer on the other side. Heights of the liquid in the two arms of the manometer were read with a cathetometer to the nearest 0.1 mm.

The amount of flow was measured between two superposed marks on the vertical body of one of the reservoirs. (An electrical timer was used, reading in seconds and tenths of a second.) At least two readings were made for each pressure setting, one emptying and one filling for the marked reservoir. The pressures used were, in centimeters of mercury: 10, 20, 30, 40, 50, 60. Since the volume of the reservoir was small relative to the volume of the storage vessel, the maximum drop in pressure was 0.01% per reading.

Commercial cocoa butter was refined in the following way: 110 g were dissolved in 150 ml of CCl_4, and 20 g of activated charcoal were added. The suspension was refluxed 1-½ hr and filtered. With a fresh 20 g aliquot of activated charcoal, the suspension was refluxed again, filtered, and placed under vacuum for solvent evaporation. The cocoa butter was re-dissolved in 150 ml of ether, 20 g of activated charcoal were added, and the suspension was refluxed 1 hr and filtered. The solution was placed over anhydrous $MgSO_4$ overnight and filtered again. Following solvent evaporation under vacuum, the still liquid fat was filtered, centrifuged, and then stored under vacuum in the centrifuge tubes.

For the experimental series, the cocoa butter was melted at 100° C. A portion was removed from the upper part of the centrifuge tube and inserted into the viscometer to fill the capillary tube and one reservoir. The viscometer was then placed in a water bath which was maintained at a constant temperature, within ± 0.05° C. At any particular temperature, 30-45 min were allowed for thermal equilibration before viscosity measurements were made. Six measurement periods were used: 1, 3, 5, 7, 9, and 13 hr. During each period, readings were made at the 6 pressures indicated above. The temperature series was as follows: 30°, 28°, 27°, 26°, 25° and 24° C. After a particular series at one temperature was made, the viscometer was placed in an oven at 100° C overnight to melt any crystals which had formed.

A syringe, with 5 ml of cocoa butter, was carried along with the viscometer through all the treatments. This 5-ml sample was used to refill the viscometer whenever the original sample suffered any diminution in volume. In addition, a microscopic chamber was subjected to the same treatments as the viscometer. During a series of measurements the chamber was kept at bath temperature on the stage of a polarizing microscope for noting the first formation of fat crystals. Because of the temperatures used, it was expected that only β-form crystals would be encountered *(11, 16)*.

RESULTS AND DISCUSSION

Calibration of the viscometer gave a value of 72 centipoises for cocoa butter viscosity at 30° C. This is to be compared with Finke's *(5)* finding of a kinematic viscosity of 70-73 centipoises at the same temperature. It was of interest that crystal nucleation did not occur within a 14-hr period at this tempearture. (However, if nucleation were induced by "tempering" at a lower temperature, slow crystal growth at 30° C could produce a few, rather large crystals.) Although no crystals were found under the microscope, the fat in the capillary had a slightly turbid appearance at the end of the 13-hr series of readings.

When the viscosity values for each pressure are averaged, regardless of the time of standing, it may be seen (Table 1) that the apparent viscosity decreases as the pressure increases. Note that this effect of "structural viscosity" is apparent at 30° C even in the absence of well-defined crystals. (The apparent viscosity in the table is given in centipoises, even though Newtonian relationships are not strictly inherent.) At lower temperatures,

TABLE 1

Apparent viscosity of cocoa butter (in centipoises) as a function of temperature and pressure

Temperature (° C)	Average pressure (Cm of Hg)					
	10	20	30	40	50	60
30	75.4	74.9	74.9	73.7	73.2	73.0
28	90.2	89.5	89.0	88.6	88.6	87.6
27	103.7	103.7	103.0	102.0	101.8	102.0
26	116.9	115.9	115.2	114.5	114.5	113.8
25	136.1	135.4	133.2	132.5	131.5	131.8
24	168.0	160.1	157.7	154.3	152.9	152.4

the successively earlier onset of crystallization results in a more marked structural viscosity because of the high concentration of crystalline particles.

The slight non-Newtonian behavior of the 30° sample must be ascribed either to inchoate crystal nuclei of other groups of molecules—of submicroscopic size—or to the form of the molecule itself. It is well-known that linear high polymers have a very pronounced structural viscosity, due to the shape of these particles. Possibly the fat molecule, in the "tuning-fork" or "chair" conformation *(2, 8)*, exhibits enough asymmetry to produce a similar viscous effect.

For the higher temperatures (*e.g.*, 30° and 28°) in which crystalline particles are not apparent for at least 8 or more hours, the amount of structural viscosity is small indeed. In these, the average drop in viscosity of a sample flowing under 60 cm Hg pressure from that under 10 cm Hg pressure is only about 3%. A graph of shear rate versus stress shows this relationship as virtually a rectilinear function. Nevertheless, the constancy of the trend demonstrates that a slight structural viscosity is a definite feature of purified cocoa butter. Even in samples at the lower temperatures, structural viscosity is evident before the onset of crystallization.

In Table 2 the structural viscosity effect has been eliminated by averaging the apparent viscosity values for all pressures at a particular time series. Here it may be seen that even when no crystallization is obvious (30° C), a continual augmentation of viscosity is a function of time.

Hinton *(7)* could find no change in apparent viscosity in chocolate at the initiation of crystallization but could detect differences when crystal nuclei had formed. It should be noted that the suspended mass of material

TABLE 2

Apparent viscosity of cocoa butter (in centipoises) as a function of temperature and time of standing

Temperature (° C)	Average time (Hours)					
	1	3	5	7	9	13
30	72.2	72.7	74.2	74.9	75.4	75.4
28	86.4	88.3	88.8	89.5	89.5*	91.0
27	98.6	102.0	102.5	103.0	103.4*	106.1
26	111.4	112.6*	113.0	114.7	117.1	121.7
25	124.1	126.5*	126.5	131.5	143.3	148.6
24	138.0*	139.9	140.2	153.4	164.6	209.3

* First noticeable appearance of crystals (polarizing microscope).

in chocolate is already so great in its viscous influence *(14)*, that the extra effect of initial fat nucleation could not be expected to be apparent.

Table 2 also shows the strong dependence of viscosity upon temperature and the extremely marked effect of large masses of fat crystals at lower temperatures. (The time of occurrence of nucleation is indicated by the position of the asterisks in the table.)

For the continual increase in viscosity with time, the only suitable explanation is that of molecular association. It is apparent that sub-nuclear molecular aggregation is occurring in the cocoa butter, to increase the volume concentration of the aggregated material. Presumably, since crystals do appear subsequently, the aggregates are in the form of submicroscopic crystal nuclei. However, the possibility that other types of aggregation may also exist cannot be excluded. Further studies have been undertaken to explore this point.

SUMMARY

Purified cocoa butter is characterized by a slight structural viscosity, even in the absence of suspended particles. As fat crystals form in the cocoa butter, the structural viscosity effect is enhanced. Sub-microscopic aggregation of fat molecules during standing of the cocoa butter is evident by the augmentation of the viscous "coefficient." This effect is apparent before the formation of microscopically visible fat crystals.

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KINETICS OF COLOR, ASCORBIC ACID, AND TOTAL ACID DIFFUSION IN FROZEN SIRUP-PACKED RASPBERRIES

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Previous work has shown that one of the best criteria for estimating temperature history in frozen raspberries is the diffusion of color from berries to surrounding sirup. Procedures for color measurement and the relationship between color and acid diffusion and time-temperature exposure have been reported *(1, 2)*. Careful analysis of extensive color and acid data suggested that changes in these naturally occurring constituents followed a process explainable on the basis of a simple kinetic theory. This paper shows that experimental data from commercial packs of berries follow the theory reasonably well, and that equations derived from this theory can be useful in assessing equivalent temperature history.

Theory. Examination of several hundred samples of commercially and experimentally sirup-packed frozen raspberries has consistently revealed that the ratio of color in berries to that in sirup decreases in essentially exponential fashion (at temperatures above 0° F) from a relatively high initial value to an equilibrium value in the neighborhood of unity. As shown in

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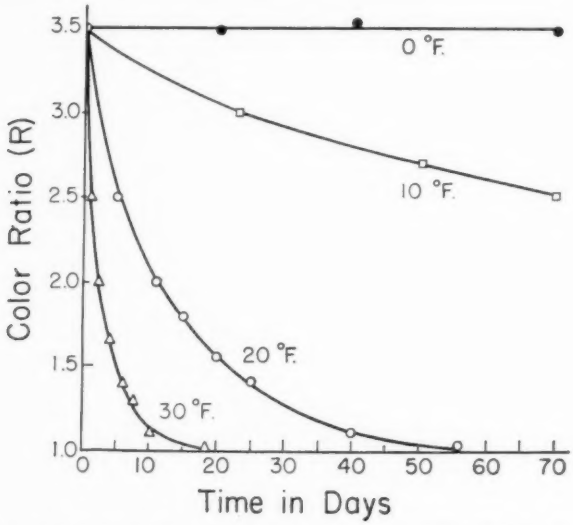

Figure 1. Effect of time and temperature on color ratio (fruit/sirup) in frozen raspberries.

the example, Figure 1, this appears to follow a first order process. Actually, the color ratio at the instant that berries and sirup are first put together is infinite, since essentially all the color is still in the berries. Obviously a certain amount of color diffuses into the sirup before the contents are frozen to 0° F or lower; this amount will depend on delay between siruping and freezing, rate of freezing, maturity of berries, degree of breakage, etc. The significance of this prefreezing diffusion will be discussed later.

If we assume a reversible process where A gives B with a first order rate constant k_1, and B produces A with a first order rate constant k_2, we may write:

$$A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} B$$

where A = color in berries and B = color in sirup at any time τ. If we let $A_o = A + B$,

$$\frac{dA}{d\tau} = k_2 A_o - k_1 A \qquad (1)$$

Straightforward integration and simplification yields the equation:

$$\ln \frac{A_o}{A - \frac{k_2}{k_1} B} = (k_1 + k_2)\tau \qquad (2)$$

If we define R as the ratio $\frac{A}{B}$, and R_e as the ratio $\frac{A}{B}$ at equilibrium,

$$R_e = \frac{k_2}{k_1} \qquad (3)$$

Now if we substitute R and R_e in equation (2), we find:

$$\ln \frac{R + 1}{R - R_e} = (k_1 + k_2)\tau \qquad (4)$$

For color distribution $R_e = 1$. Let $k = k_1 + k_2$. Now if we let $\tau = a + t$, where a represents effective time between introduction of sirup to berries and initial color ratio measurement, and t represents time at a known steady temperature after the initial measurement, the equation becomes:

$$\ln \frac{R + 1}{R - 1} = k\,(a + t) \qquad (5)$$

Since previous work on raspberries *(1)* has shown the advantage of expressing color distribution as a ratio, all of our data are in this form.

Figure 2 shows that equation (5) holds for temperatures from 10° to 30° F. The slopes of the straight lines give the values of k. The intercepts on the time axis give the value of a (in days) that would be required at each of the temperatures to give the color ratio found at zero time. Alpha is not zero, since, as mentioned earlier, a certain amount of diffusion occurs before the package is originally frozen to 0° F or lower. Thus, at 30° F it would have taken 2.9 days; at 25°, 5.3 days; at 20°, 12.8 days; at 15°,

67 days; and at 10°, 164 days to reach the color ratio found at zero time if we had been able to mix and freeze the sample with no transfer of color until it reached these temperatures.

Since the values shown in Figure 2 were obtained from a fairly uniform lot of raw material which was randomly divided and placed at the various temperatures, the amount of prefreezing diffusion was the same for all temperatures. Hence the value of a for each of these temperatures is a measure of the rate of change in color ratio at that temperature and is inversely related to the rate constant k.

Practical application. In order to make practical use of equation (5) we must first determine the range of a values that could be reasonably expected when proper commercial processing and freezing procedures are employed. Obviously, even under the best commercial conditions, a variable amount of color will be transferred from berries to sirup before the mixture is frozen. It is also evident that an additional amount of color will be transferred to the sirup during thawing in preparation for the color determinations. The range of a values was determined on 20 commercial lots of sirup-packed raspberries stored at a constant 20° F.

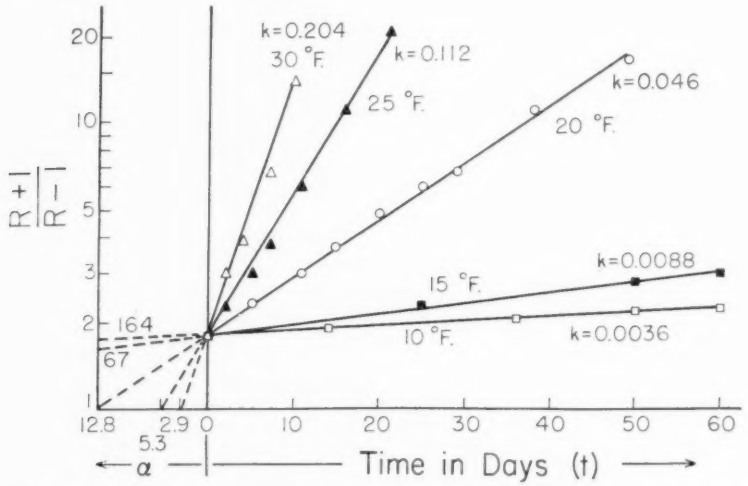

Figure 2. Effect of temperature on the rate constant k and corresponding a value when observed color ratios are substituted for R in the expression $R + 1/R - 1$.

Figure 3 shows a plot of the ratio $R + 1/R - 1$ against time on semilogarithmic paper. It should be noted that even though the zero time $R + 1/R - 1$ values ranged from 1.3 to 1.9, the slope of the lines or rate constant k remained essentially constant. The top and bottom lines represent the extremes in zero time values for the 20 different lots while the center line represents the average. This yields an average a value of 9.5 ± 3.5 days for a steady reference temperature of 20° F. It is recognized that some commercial samples may not fall within this range of a values because of overmaturity, undue

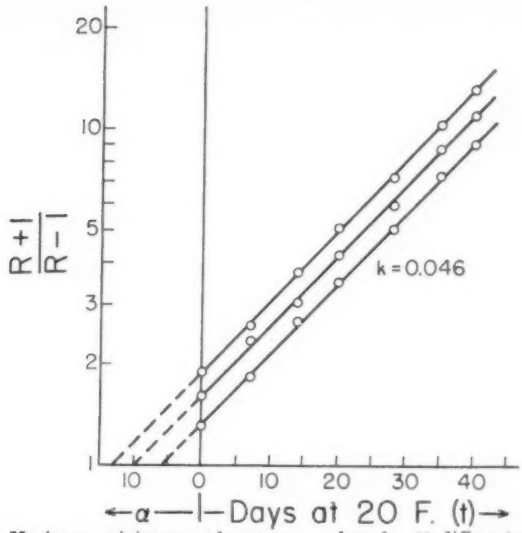

Figure 3. Maximum, minimum, and average α values for 20 different commercial lots of frozen raspberries stored at 20° F.

breakage of berries, exceptionally long delays prior to freezing, etc. These factors which definitely reduce the overall quality of the pack would tend to give higher α values than those reported above. However, since holding at unfavorable temperatures also gives higher α values and reduces quality, it is not too important whether the high α values occur as a result of quality damage prior or subsequent to freezing. The important point is that the k value at a given temperature remains essentially constant over a wide range of variability in amount of prefreezing diffusion (α value).

In addition to the lots of commercial samples used to establish the limits shown in Figure 3, 14 additional case lots of berries were randomly selected from various freezing plants and placed in storage at 20° F. At periodic intervals samples from each case lot were removed and analyzed for color ratio. The $R + 1/R - 1$ values for all of these samples were well within the limits shown in Figure 3 for each storage interval up to 40 days. This provides additional evidence that the limits established in Figure 3 are large enough to encompass a wide range of commercial samples.

From the established values of α and k, equivalent temperature history for any given color ratio can be simply determined by solving equation (5) for t. If these determinations were necessary on a routine basis, it would obviously be more convenient to construct a curve relating color ratio with time, and read temperature history directly from the curve.

Application to other measurements. Since the experimentally determined rates and extent of color diffusion between berries and sirup satisfactorily fit the theory outlined in this paper, it was of interest to apply the theory to similar measurements such as total acid and ascorbic acid ratios. In both instances, as in color measurement, the ratio at the instant that berries and sirup are placed together is infinite. Both of these ratios also

decline and asymptotically approach an equilibrium value in essentially exponential fashion, much like the curves shown for color ratio in Figure 1. However, as pointed out in a previous publication *(2)*, the total and ascorbic acid equilibrium values in the temperature range of 0° to 30° F fall below unity. Several measurements on the berries used in these experiments indicated that total acid ratio remains essentially constant at 0.7 and ascorbic acid ratio is fairly constant at a value of about 0.5. The basic process for all three measurements is very similar; namely, the diffusion of color, total acid or ascorbic acid from berries to sirup and vice versa. The only apparent difference lies in the fact that the equilibrium values for the three measurements differ. Thus, the value of R_e in equation (4) should be 0.7 if we are dealing with total acid ratios, and 0.5 if ascorbic acid ratios are being used. For total acid ratios the equation would then become

$$\ln \frac{R+1}{R-0.7} = k\,(t+a) \tag{6}$$

and for ascorbic acid ratios it would be

$$\ln \frac{R+1}{R-0.5} = k\,(t+a) \tag{7}$$

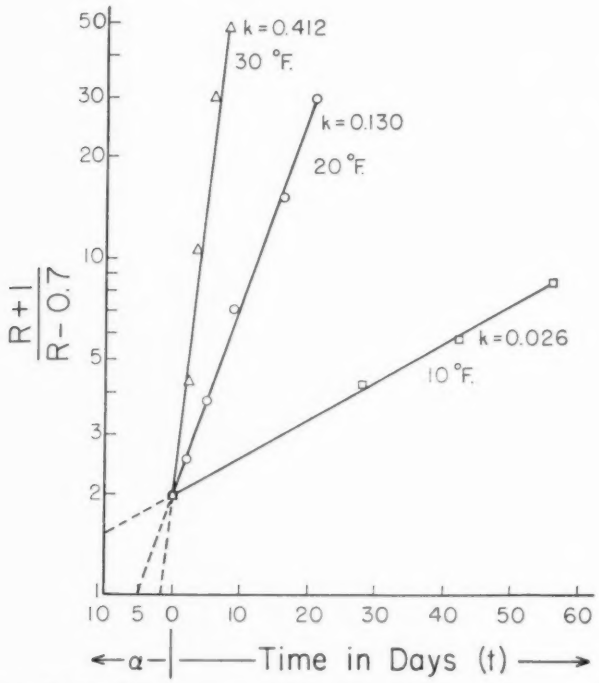

Figure 4. Relationship between (t) and the expression R + 1/R — 0.7 when total acid ratio is substituted for R.

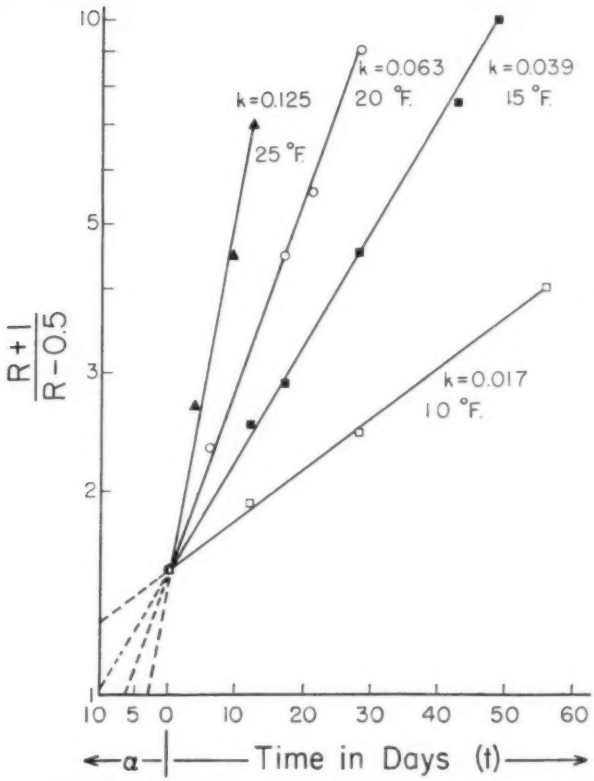

Figure 5. Relationship between (t) and the expression R + 1/R — 0.5 when ascorbic acid ratio is substituted for R.

Now if we plot the logarithm of the ratio $\frac{R+1}{R-0.7}$ and $\frac{R+1}{R-0.5}$ against time at various temperatures from 10° to 30° F., we should obtain straight lines for both measurements. Figures 4 and 5 show that the logarithm of both these ratios is indeed a linear function of time in this temperature range. The same principles discussed in connection with use of a values and rate constants for estimation of temperature history from color measurements would apply to equations (6) and (7). Therefore, the rate constants and a values obtained from equations (6) and (7) can be used in the same way as those obtained with color ratios.

When the rate constants obtained for color, total acid, and ascorbic acid diffusion were plotted against the reciprocal of the absolute temperature, these measurements appeared to follow the Arrhenius equation in the 10° to 30° F temperature range. Although the data do not permit accurate

determination of activation energies, the apparent values in kilocalories over this temperature range were of the order of magnitude of 60 for color diffusion and 37 for both total acid and ascorbic acid diffusion.

SUMMARY

A simple kinetic theory for the distribution of color and acid between raspberries and sirup is postulated. The diffusion of these constituents follows a first order process with rate constants which appear to obey the Arrhenius equation. An equation which fits experimental data obtained from commercial samples of frozen berries is derived. Various parameters in the equation are discussed with respect to their significance in the estimation of temperature history in frozen raspberries.

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COLOR MEASUREMENT AND DETERIORATION IN GRAPE AND BERRY JUICES AND CONCENTRATES

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(Manuscript received July 13, 1959)

Red color is an important index of quality in juices and concentrates made from red-pigmented berries and grapes. High-quality products prepared under conditions of minimum heat damage retain their natural red color. This natural red color is attributed to anthocyanin pigments. Heat-damaged products assume a dull brown color as deterioration proceeds. This color change is attributed to a decrease in anthocyanin pigments accompanied by an increase in brown products of one or more reactions *(2, 6, 12, 15)*.

Loss of natural red color by processed fruit products has been shown to be hastened by such factors as presence of certain metallic cations *(1)*, actions of enzymes *(5)*, oxygen *(12, 19)*, ascorbic acid *(11, 19)*, and high temperatures during processing and storage *(2, 6, 9, 11, 12, 13, 14, 15)*. Studies on anthocyanin pigments extracted from fruits bear out in general the observations made on fruit products *(8, 10, 11, 18, 19)* although changes may be more striking in solutions of pigments than in the products *(15)*. Increase in brown color is also an important deteriorative process which apparently proceeds independently of loss of anthocyanin pigment *(2, 6, 9, 13, 14)* and is variously reported to precede *(9)* or to follow *(7)* the destruction of anthocyanins.

The method of Sondheimer and Kertez *(17)* has been widely used to determine the amount of red pigment in fruit products. It is based upon a comparison between a solution of Congo Red and a solution of the pigment from the product. As pointed out by Francis *(4)*, some error is introduced by variability in different lots of the dye. Red color is frequently measured by optical density at the wavelength at which an absorption maximum is detected *(10, 12, 13, 15)*. The importance of measuring optical density at two wavelengths to detect increase in browning (400 to 440 mmc) and fading of red (500 to 535 mmc) has been pointed out *(13, 15)*, and Lukton *et al (8)* used the ratio of densities at 500 and 400 mmc to evaluate pigment degradation.

Thermal degradation of anthocyanin pigment in strawberry jelly has been shown to be a logarithmic function of the time of heating at a given temperature *(2)*. The half-life of the pigment in solution *(10)* or in preserves *(11)* has likewise been shown to be a logarithmic function of temperature.

The work reported here includes studies on temperature conditions and rates at which color of red-pigmented juices changes perceptibly, and a means of evaluating the color and color changes of a given product during processing or storage.

EXPERIMENTAL

Preparation of juices and concentrates. Juices were prepared from *Vitus vinifera* grapes, boysenberries and strawberries. Grape juices were from Zinfandel, Carignane,

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and Alicante Bouschet varieties. The grapes were stemmed, crushed, and then heated to 160° F for 5 min in a steam-jacketed kettle and pressed hot. The cooled press juice was filtered brilliantly clear in a pressure filter after adding a small amount (¼ to ½%) of a diatomaceous earth filter-aid. A blend of the grape juices was prepared: Alicante 45%, Carignane 45%, Zinfandel 10%. This blend is typical of vinifera grape juice produced commercially.

Boysenberry and strawberry juices were prepared by comminuting the berries, treating the puree with a commercial pectin-hydrolyzing enzyme preparation, pressing cold in a hydraulic bag press after adding 2 to 4% of filter-aid, and filtering clear in a pressure filter as described above.

Six-fold boysenberry concentrates were prepared in vacuum concentration equipment at about 100° F. At this low temperature there was no detectable loss in color during concentration.

Measurement of color. The absorption spectra of the juices were measured on a Cary Model 14 recording spectrophotometer and a Beckman model DU spectrophotometer.[b] The juices were diluted with water or with 0.25 *N* HCl. At dilutions of 100 : 1 and 250 : 1 in 0.25 *N* HCl the pH was sufficiently low to ensure the maximum color intensity. At this low pH the color develops immediately with grape and boysenberry juices. Strawberry pigment has a different absorption maximum and the color does not reach maximum intensity for 2 to 3 hr in 0.25 *N* HCl; a longer time is required at higher pH. Measurements of strawberry color were made after 3 hours' standing.

For routine color determinations, the absorbancy (optical density) was measured in an Evelyn colorimeter. Grape and boysenberry absorbancies were measured at the absorption maximum of 520 mmc and at 420 mmc. Strawberry color was measured at 490 and 420 mmc. Color of concentrates was measured in the same manner as for juices after equivalent dilution with 0.25 *N* HCl.

Color difference measurements. The light transmitted through samples 1 cm deep was measured with the Hunter Color and Color Difference Meter. The same samples were observed visually. The meter was set to give the following readings with water in the 1 cm sample cell: L = 100, a = 0, b = 0. Various dilutions of the strawberry, grape, and boysenberry juices were compared with the original juice to determine at what dilution a difference could be seen and/or measured with the meter. A 90% sample contained 90% of the juice and 10% water, etc. Results with the Hunter meter are expressed as total color difference, E, according to the equation derived by Scofield *(16)*:

$$\Delta E = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2}$$

Effect of time-temperature conditions on color. One-ml samples of juice were sealed in 10 x 75 mm test tubes and the tubes were immersed in oil baths at various temperatures for different lengths of time. The tubes were cooled in a water bath and the contents transferred to a volumetric flask with 0.25 *N* HCl.

Absorption spectra (Figure 1) or absorbancy at selected wavelengths (Table 1) were measured on samples of juice diluted with water. For the purpose of determining the conditions under which 10% of the red color of a juice is lost, absorbancy of 520 mmc (grape, boysenberry) or 490 mmc (strawberry) was measured on samples which had been heated for different lengths of time at each of several temperatures. A 10% loss in red color was selected because it is a visually perceptible change, as explained below. These data were plotted to give several absorbancy vs time curves from each of which the time for 10% decrease in absorbancy was noted. The figures thus obtained for 10% color loss are plotted in Figure 3.

Boysenberry concentrate was canned at 190° F, cooled, and samples were stored at −10° F, 40° F, and 70° F. Absorption spectra were determined.

RESULTS AND DISCUSSION

The visible absorption spectra in Figure 1 are from grape and boysenberry juices diluted with water and measured with a spectrophotometer employing 1-cm cells. The juices all show absorption maxima at 520 mmc,

[b] Mention of commercial products does not constitute endorsement by the U. S. Department of Agriculture over others of a similar nature not mentioned.

TABLE 1

Effect of heating at 100° C on color of juices

	Absorbancy														
	Grape Blend 133:1 [1]			Alicante Bouschet 133:1 [1]			Zinfandel 133:1 [1]			Carignane 133:1 [1]			Boysenberry 250:1 [1]		
Hours at 100° C	Wavelength millimicrons		Ratio	mmc		Ratio	mmc		Ratio	mmc		Ratio	mmc		Ratio
	520	420		520	420		520	420		520	420		520	420	
0	0.449	0.175	2.56	0.589	0.226	2.61	0.653	0.240	2.72	0.171	0.072	2.38	0.502	0.191	2.63
1	.260	.123	2.11	.278	.140	1.99	.263	.149	1.77	.077	.047	1.64	.337	.135	2.50
2	.171	.103	1.66	.204	.126	1.62	.155	.158	0.98	.047	.051	0.92	.246	.101	2.44
3	.140	.105	1.33	.146	.121	1.21	.128	.204	0.63	.045	.062	0.73	.152	.080	1.90
4	.118	.123	0.96	.111	.140	0.79	.114	.240	0.48	.036	.094	0.38	.108	.063	1.71

[1] Dilutions with 0.25 N HCl for colorimetric measurements.

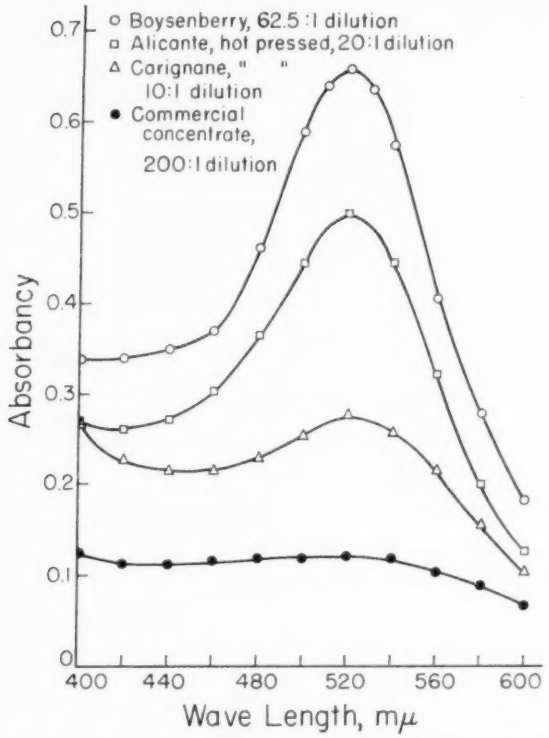

Figure 1. Absorption spectra of juices and a commercial grape juice concentrate.

although several anthocyanin pigments are reported to be present (Dupuy and Puisais, *3*). Fourteen grape varieties tested showed absorption maxima at or near 520 mmc. The spectrum of a sample of commercial red grape concentrate is included. All methods of processing do not cause such a pronounced decrease in the red color; vacuum-concentrated juices prepared in the laboratory have spectra virtually indistinguishable from those of fresh juices.

The effect of heat upon the red pigment is further illustrated in Figure *2*. In this experiment Zinfandel grape juice was heated 1 and 2 hr in a boiling water bath in sealed tubes. The samples were then diluted 166:1 with 0.25 *N* HCl and absorption spectra were obtained with a spectrophotometer employing 1 cm cells. The spectrum of a low-quality commercial juice sample diluted 133:1 with 0.25 *N* HCl is shown for comparison. It is apparent that the first effect of heating on color is to cause it to fade; that is, the absorbancy merely decreases, with no shift in wavelength of the absorption maximum and no increase in absorbancy at other wavelengths. Upon prolonged heating the juice gradually turns brown, accompanied by an increase in absorption in the region of 400 to 460 mmc (Figure *2*).

The fact that heat damage causes a decrease in absorption at one wavelength (520 mmc) due to the loss of natural pigment and an increase in absorption at another wavelength (420 mmc) due to browning suggests that a measure of the extent of heat damage can be obtained from the ratio of these two absorbancy values, as noted by other workers *(8, 13, 16)*. In a fresh juice the ratio of absorbancies, $\frac{A_{520\ mmc}}{A_{420\ mmc}}$, is higher than in the heat-damaged juice. The ratio of absorbancies at 520 mmc and 420 mmc was satisfactory for the red viniferous grape juices and boysenberry juice in indicating severe heat damage. The ratio of absorbancies at 490 mmc and 420 mmc was used for strawberry juice, and other ratios would apply for juices having different absorption maxima.

The ratios of absorbancies at 520 and 420 mmc of boysenberry and grape juices heated for various lengths of time at 100° C are set forth in Table 1. Absorbancies at both wavelengths decrease rapidly at first as the red color of the juice fades. Then absorbancy at 420 mmc tends to increase as the juices become brown. Thus the ratio A_{520}/A_{420} decreases constantly as heat damage progresses, which shows it to be a useful indication of heat damage in these juices. The point in time where the ratio of absorbancies is one, was found to be near the point where browning became visible. There is variation among different types of juice and different colorimeters. Good quality grape and boysenberry juices measured with the Evelyn colorimeter

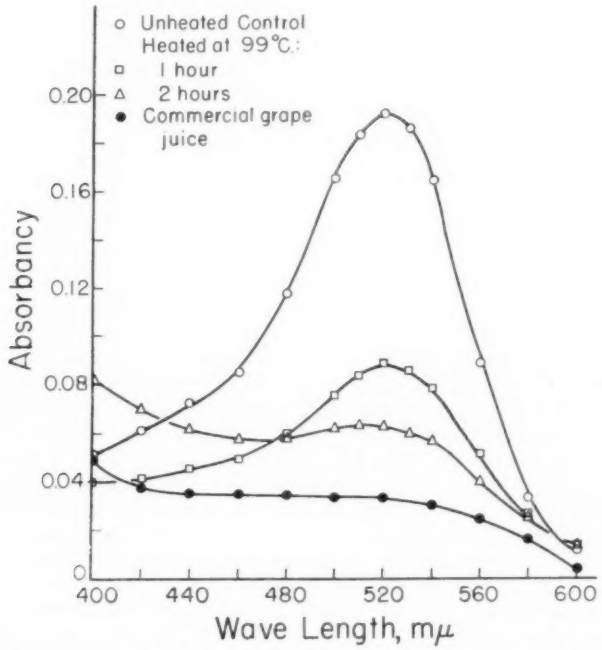

Figure 2. Absorption spectra of Zinfandel grape juice heated for various times.

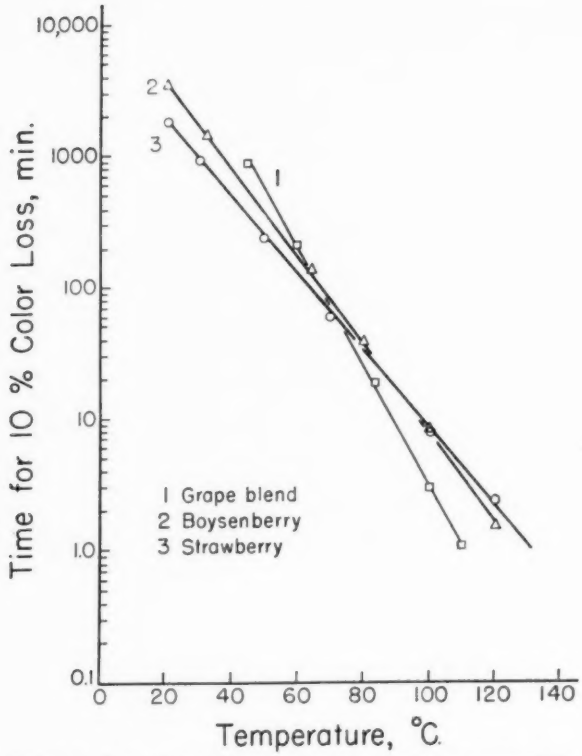

Figure 3. Rate of loss of 10% of color at various temperatures.

had a ratio of 2.4 or above (3 or above on the Beckman spectrophotometer). Severely heat-damaged grape juices had ratios of 1 or below, while similarly treated boysenberry juice had a ratio of 1.7.

The stability of red-colored juices and anthocyanin pigments has been variously expressed in terms of half-life of the color *(2, 10, 11)*, as constants for rate of loss of color *(8, 18)*, and as changes in light transmittance *(12)* or optical density *(13, 15)*. To determine what differences in color could be perceived, a series of dilutions of boysenberry juice and grape juice were prepared. The juices and diluted samples were observed by transmitted light through cells 1 cm deep. The samples in the same cells were presented to the Hunter Color and Color Difference meter for measurements of light transmission and color differences. A panel of 10 judges correctly distinguished single-strength boysenberry juice from a sample diluted to contain 92% juice; the judges failed to distinguish a 96% juice from the single-strength sample. The color difference between the 92% sample and the original juice expressed as ΔE was 1.7. In grape juice a 93% sample was distinguished from the original; ΔE for this pair was 3.6. Strawberry juice diluted to 90% was easily distinguished from the original and the ΔE was

6.3; the limit of perceptible differences was not determined for strawberry. It was concluded that a 10% loss in red color was easily perceptible in these juices if viewed through a 1-cm depth as above.

The rates of thermal degradation of these juices was determined in terms of the length of time necessary to produce a 10% loss in red color as measured at their absorption maxima. These data are shown in Figure 3, from which it can be seen that the time necessary for a 10% color loss is a logarithmic function of the temperature at which the juice is held. The Q_{10} values of the changes measured here (number of fold change in rate for a change in temperature of 10° C) are: strawberry 2; boysenberry 2.1; grape 2.7. These data suggest that grape juice will retain its color somewhat better at room temperature than will strawberry and boysenberry juices. Refrigeration during storage would be more necessary for strawberry and boysenberry juices than for grape juice, although low-temperature storage will retard color deterioration in any of the juices.

The effect of storage at −10°, 40°, and 70° F on color of boysenberry concentrate is shown in Figure 4. The loss in red color during storage as

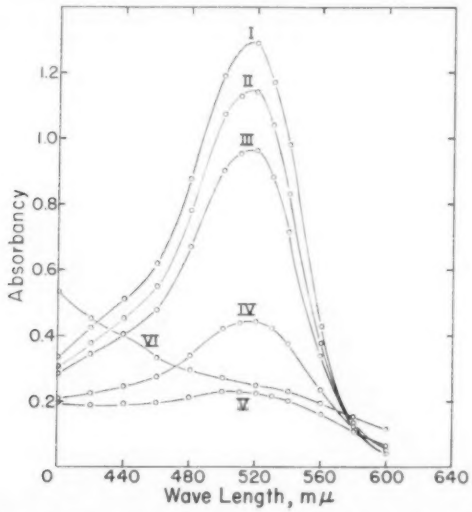

Figure 4. Absorption spectra of Boysenberry juice concentrates diluted 250:1 with 0.25 N HCl. I. Control −10° F. II. 6 months at 40° F. III. 12 months at 40° F. IV. 6 months at 70° F. V. 12 months at 70° F. VI. 3 hours at 212° F.

detected by measuring absorbancy at 520 mmc is obvious. The sample stored 12 months at 70° F was considered red when viewed alone. However, when viewed in comparison with the control sample, it was obviously less red, and a brown component could be discerned. The sample heated 3 hours at 212° F (curve VI, Figure 4) was brown; as can be seen from the absorption spectrum the ratio $\frac{A_{520}}{A_{420}}$ is about 0.5. This represents a more rapid deterioration of color than was shown for single-strength juice (Table 1).

SUMMARY

The color characteristics of grape, strawberry, and boysenberry juices were determined by spectrophotometric measurements. Thermal degradation of the red color of these juices is expressed in terms of time necessary for a 10% decrease in absorbancy at the absorption maximum of each juice. This increment of color loss is discernible by visual comparison of samples 1-cm deep. The absorption spectra of boysenberry concentrates stored at different temperatures illustrated a progressive change in color from red to brown.

Acknowledgment

The assistance and advice of Mr. H. C. Lukens in conducting color measurements is gratefully acknowledged.

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NUTRITIVE VALUE OF FISH.[a] II. BIOTIN, FOLIC ACID, PANTOTHENIC ACID, AND FREE AMINO ACIDS OF VARIOUS SALT-WATER SPECIES

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(Manuscript received August 13, 1959)

In a previous report *(3)* information concerning the nicotinic acid, riboflavin, vitamin B_{12}, and protein amino acids of various salt-water species commonly consumed in New England and other areas was presented. Similar information with respect to biotin, folic acid, pantothenic acid, and free amino acids is reported herein.

EXPERIMENTAL PROCEDURE

Market-fresh samples of various species of fish, including shellfish, commonly consumed in New England, were analyzed quantitatively for biotin, folic acid and pantothenic acid, and qualitatively for free amino acids. Some canned samples were also analyzed.

Edible portions of fish muscle were blended and extracted as previously described *(3)*. Vitamin analyses were carried out by standard quantitative microbiological procedure *(1)*, using *Lactobacillus arabinosus* 17–5 (A.T.C.C. No. 8014) for biotin and pantothenic acid, and *Streptococcus lactis* R (A.T.C.C. No. 8043) for folic acid.

To determine the free amino acids occurring in the tissue, a portion of blended fish was successively extracted with water, by use of the centrifuge; and the water extracts were combined, and concentrated by heating. The insoluble protein which separated out was removed by centrifuging. Traces of protein remaining in the solution were removed with lead acetate, and the excess lead was removed with sodium oxalate. Aliquots of the water extract so prepared, and adjusted to a definite volume, were subjected to one and two directional chromatography. Other aliquots were subjected also to hydrolysis by refluxing at 100° C for 24 hr in a final concentration of 1–1 HCl. The excess HCl was removed with Amberlite IR-4B (OH). The solution was concentrated, the volume adjusted, and aliquots chromatographed.

The chromatographic methods used were the same as those previously reported *(3)*. Insofar as possible, the chromatographic spots were identified by their location and by their reactions to various tests.

Individual areas on the chromatograms were tested for the possible presence of peptides. To accomplish this, large aliquots of the water extract were chromatographed in duplicate, using the two directional method. One sheet was sprayed with ninhydrin to locate the spot. The corresponding area on the second chromatogram was cut out and the material eluted with water. The eluate was concentrated to a small volume, of which one aliquot was chromatographed directly and another was chromatographed after being subjected to hydrolysis in the same manner as previously noted, except that the excess HCl was removed by repeated evaporations.

RESULTS AND DISCUSSIONS

Average results of the biotin, folic acid and pantothenic acid analyses are presented in Table 1. As has been reported previously for other vitamins *(3)* and species *(2)*, a rather wide variation of results within a species was found in some instances. Examples are biotin in salmon (2.4–20.8 milli-

[a] Published with the approval of the Director of the New Hampshire Agricultural Experiment Station as Scientific Contribution No. 247.

mcg/g), and smelts (12–48); and folic acid in cusk (6.2–46.0 milli-mcg/g), halibut (7.5–50.4), salmon (13.2–45.0), smelts (20.4–63.0), clams (12.8–65.0), and shrimp (49.2–132.0).

Results indicate in general that many marine species have a relatively high nutritive value with respect to the vitamins studied. The particularly good sources of biotin include haddock roe, lobsters, crabs, smelts and clams. Several other species could be classed as good sources. Haddock roe is an exceptionally good source of folic acid. Other species containing relatively large amounts of this vitamin include shrimp, smelts, oysters,

TABLE 1

Average B vitamin content of various species (fresh weight basis) [1]

Species	Biotin	Folic acid	Panthothenic acid [2]
	Milli-Mcg/g	Milli-Mcg/g	Mcg/g
Cod	1.89	17.5	1.22
Cusk	1.17	19.8	1.15
Haddock	2.93	8.13	1.40
Haddock Roe	122.8	637.	36.2
Halibut	18.9	19.5	2.99
Mackerel	15.0	12.4	4.64
Salmon	8.52	21.5	7.45
Smelts	30.3	36.6	6.38
Swordfish	1.61	20.4	2.03
Molluscs:			
Clams	23.4	26.5	6.22
Oysters	7.23	37.0	1.84
Scallops	3.16	5.08	1.43
Crustaceans:			
Lobster (claw)	51.8	5.48	19.9
Lobster (tail)	47.9	5.63	12.7
Shrimp	10.1	73.8	3.72
Canned fish:			
Crab	46.0	3.0	4.9
Salmon	14.8	6.7	5.7
Tuna	4.5	32.5	2.0

[1] Each figure represents 6 to 15 samples.
[2] Calculated as calcium pantothenate.

clams and tuna. The best sources of pantothenic acid are haddock roe and lobsters, but fairly high amounts of this vitamin are found in all species. In view of these results, together with those previously reported for nicotinic acid, riboflavin and vitamin B_{12} *(3)*, it can be concluded that, in general, the marine species studied are good sources of B– complex vitamins.

Table 2 shows the distribution of free amino acids as found in the water extracts of fish muscle. The most prominent ones consistently present in all species were glutamic acid, glycine, alanine, taurine, valine and leucine. B-alanine was commonly present. Histidine was present in a particularly large amount in mackerel.

TABLE 2

Free amino acids in water extracts of various species[1]

	Cod	Cusk	Haddock	Halibut	Mackerel	Salmon	Smelts	Swordfish
Glutamic acid	+	+	+	+	+	+	+	+
Glycine	+	+	+	+	+	+	+	+
Taurine	+	+	+	+	+	+	+	+
Alanine	+	+	+	+	+	+	+	+
Valine	+	+	+	+	+	+	+	+
Leucine	+	+	+	+	+	+	+	+
Beta alanine	+	+	+	+	low	+	low	+
Histidine			+	+	+	+	+	+
Serine			+	+	+	+	+	+
Threonine			+	+	+	+	+	+
Arginine					+		+	
Methionine					+		+	
Phenyl alanine					+		+	
Aspartic acid					+		+	
Proline					+		+	
OH-Proline								
Tryptophan								
Lysine								
Cystine								
Tyrosine								

[1] + = present.

After hydrolysis of the water extracts, amino acids in addition to those present before hydrolysis were evident, indicating the presence of some peptides in the water extracts. For example, histidine appeared in cusk; serine and threonine in cod; and arginine, proline, lysine and aspartic acid in most species where previously absent. Phenylalanine and hydroxyproline appeared in halibut, salmon, and swordfish. Hydroxyproline also appeared in cod. A heavy guanidine spot appeared on the chromatograms of the hydrolyzed water extracts of all species, and the spots for glycine, taurine, histidine and β-alanine were much more prominent than with the unhydrolyzed extracts. A heavy yellow ninhydrin reacting spot which was present on the chromatograms of the unhydrolyzed water extracts of all species except mackerel disappeared, in all instances, upon hydrolysis.

The heavy yellow ninhydrin reacting spot is apparently due in part to the dipeptide anserine (β-alanyl-1-methyl-L-histidine). This conclusion is based on the fact that upon hydrolysis and subsequent analysis of the eluate from this area, the chromatograms consistently showed the presence of both β-alanine and a ninhydrin reacting spot which turned green at a temperature over 110° C and which was consistent chromatographically with methyl histidine.

These chromatograms also showed a pronounced spot reacting positively to the Sakaguchi and alkaline ferricyanide-nitroprusside tests for the guanidine group. This compound was found to be consistent chromatographically with methyl guanidine.

Further investigation revealed the presence of creatine and creatinine in the water extracts of the various species. Each of these compounds when subjected to the hydrolysis procedure used in this work gives a chromatogram spot coincident with that of methyl guanidine. Since the chromato-

graphic location of creatine is in the same area as the yellow ninhydrin spot, undoubtedly it is cut out and eluted with the latter and so accounts for the guanidine test on hydrolysis.

An unidentified spot occurred, on the two directional chromatograms of the unhydrolyzed extracts, lower than B-alanine and alanine in the collidine-lutidine direction and between them in the phenol direction. It could be removed with IRC-50 cation exchange resin, and was missing after hydrolisis of the samples. After hydrolysis the amino acids lysine and arginine appeared, indicating the original presence of a water-soluble peptide containing these basic amino acids.

Shellfish were also included in this survey. Analyses were carried out in the same manner as with the fish. The most prominent amino acids in the water extracts included glutamic acid, glycine, taurine, alanine, valine, leucine, arginine and proline. Glycine and arginine were present in particularly high concentrations. Table 3 shows the free amino acid distribution

TABLE 3

Free amino acids in water extracts of various shellfish [1]

	Scallops	Clams	Oysters	Shrimp	Lobster (tail)
Glutamic acid	+	+	+	+	+
Glycine	+	+	+	+	+
Taurine	+	+	+	+	+
Alanine	+	+	+	+	+
Valine	+	+	+	+	+
Leucine	+	+	+	+	+
Beta alanine	?	+	+		?
Histidine		+			
Serine		+	+	?	+
Threonine		+	+	?	+
Arginine	+	+	+	+	+
Methionine		+			
Phenyl alanine		+	+	?	
Aspartic acid	?	+	+	+	+
Proline	+	+	+	+	+
OH-Proline				+	+
Tryptophan		+		+	
Lysine		?		+	+
Cystine					
Tyrosine		+	+	+	+

[1] + = present.
? = probable trace.

in these species. In contrast to the vertebrate fish, the shellfish did not show the yellow ninhydrin spot described above. Little change occurred upon hydrolysis of the water extracts, indicating an absence of water-soluble peptides.

SUMMARY

Various species of salt-water fish were analyzed quantitatively for biotin, folic acid and pantothenic acid, and qualitatively for free amino acids. In general, these species are valuable dietary sources of the vitamins studied.

A distribution of free amino acids occurs in fish muscle. Certain amino

acids are consistently present, and there is a variation among species in the occurrence of others. A unique difference is noted in mackerel; namely, the absence of the dipeptide anserine which occurs in all the other species studied. Since upon hydrolysis of the water extracts of most species the amino acid distribution shows a greater variety and a higher concentration, the presence of several water-soluble peptides in the fish muscle is indicated.

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THE BROWNING OF ASCORBIC ACID

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(Manuscript received May 21, 1959)

The darkening of citrus juices has been studied extensively, and it has been shown that when a citrus juice darkens carbon dioxide is evolved and ascorbic acid is lost *(3, 6, 5)*. On the basis of the chemical structure of ascorbic acid, it has also been postulated that in these fruit juice systems the vitamin C reacts with amino acids in much the same way as sugars react in the Maillard browning system *(5)*. Lahikainen *et al (7)* showed, in model systems containing ascorbic acid, amino acids, and acidic buffers at concentrations corresponding to those found in fruit juices, that amino acids were not involved in the CO_2 production during the initial phases of browning.

Lamden and Harris *(8)* investigated the browning of ascorbic acids in 50% solutions of citric acid. They observed that browning occurred rapidly at 100° C in the presence or absence of oxygen, that CO_2 was liberated with little loss of ascorbic acid, and that furfural was formed. In a paper on the browning of glucose and glycine solutions, Bohart and Carson *(1)* reported that trace metals, oxygen and light increased the rate of darkening.

In this paper, we report the effect of light and metal upon the darkening of ascorbic acid-buffer solutions of dilute concentrations.

EXPERIMENTAL

Reaction mixtures for all experiments were made in glass redistilled water using twice recrystallized ascorbic acid, citric acid and redistilled acetic acid. The base used to adjust the pH to 7 was made by dissolving sodium metal in ethanol, adding water, and distilling the ethanol.

The apparatus used for the experiments described in this paper is shown in Figure 1. The reaction tube (volume 70 ml) contained a side arm through which oxygen was bubbled during the experiment. This procedure insured the maintenance of a saturated oxygen solution during the entire reaction. The oxygen entering the reaction tube was freed of carbon dioxide by first bubbling through a sodium hydroxide trap.

The carbon dioxide evolved from the reaction mixture was swept through a reflex condenser with the stream of oxygen to a sodium hydroxide trap. An additional trap was placed down stream to prevent any back diffusion of atmospheric CO_2 into the system. At intervals the sodium hydroxide trap (one normal sodium hydroxide) immediately down stream from the reaction tube was removed, the glass sinter rinsed with CO_2 free water, and a solution of 10% barium chloride added to the tube. The contents were then titrated to phenolphthalein endpoint with HCl. This manipulation was done as quickly as possible. Previous experiments had shown that no appreciable atmospheric CO_2 was picked up by the trapping solution during the addition of barium chloride and subsequent titration. In most instances separate reaction trains were used for determining active CO_2 at the different intervals. By using a known volume of sodium hydroxide in the tube and titration with a standard hydrochloric acid solution the total amount of CO_2 evolved could be determined with only moderate error. The total evolved CO_2 was then the sum of the CO_2 evolved at the different intervals.

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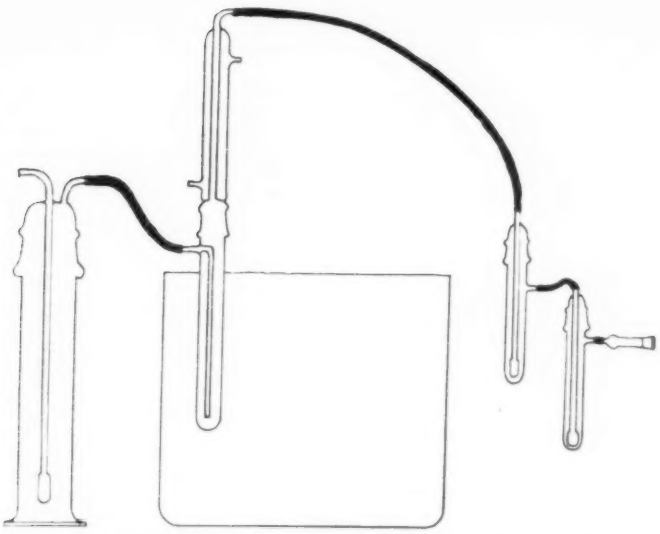

Figure 1. Apparatus for the collection of CO_2 from browning reactions.

Metal catalysis. The rate of browning of 0.025 molar ascorbic acid solutions containing a 100 ppm of added metallic ions and maintained at pH 7.0 by adding sodium acetate buffer were measured photometrically at 550 mmc. The solutions were 0.11 molar in sodium acetate buffer. In experiments with metal catalysis initial runs with the apparatus indicated that during browning a shift in pH occurred. A sample run was made to determine the shift and the amount of alkali that had to be added in order to maintain pH 7. During the run in which the optical density during browning was determined additional buffer was added by quickly opening the tube, adding the requisite amount of acid with shaking, and quickly reclosing the reaction train. The optical densities were measured at intervals by removing the reaction tube from the train and pouring its contents into a cuvette. A separate apparatus was used for each determination of optical density during the run. The pH's were adjusted as before on the tubes which were not used for measurement of optical density determinations. It should be noted here that the same tubes or the same reaction solutions were not used for the optical density measurements and the CO_2 collection so that the back diffusion or introduction of atmospheric CO_2 into the solutions used to determine optical density was of no particular significance since it would normally be swept out extremely rapidly in the stream of oxygen. The reaction vessels were maintained at 50° C by immersion in a water bath.

One hundred parts per million of iron, copper and ethylene diaminetetraacetic acid (EDTA) were added to the ascorbic acid solution just prior to their immersion in the heating bath. Figure 2 illustrates the darkening observed in these solutions as measured by means of a Beckman D.U. spectrophotometer. It is to be noted that the EDTA containing solutions darkened the most rapidly and achieved the highest optical density. Repeated experiments with this compound indicated that residual amine groups present in the preparation were directly correlatable with the amount of browning observed. The larger the percentage of free amines, the more rapidly browning took place.

Although the copper and iron containing solutions lost the free ascorbic acid extremely rapidly compared to the control, they did not darken as rapidly as the solutions containing no significant metal concentration.

Light catalysis. Bohart and Carson *(1)* found that light accelerated the browning of a Maillard solution. To test the light effect upon the browning of ascorbic acid buffer

solutions, their browning was followed in complete darkness, under illumination by diffuse daylight, and in alternate darkness and illumination. In the latter experiment, a 275 sunlamp was placed 1 ft from the constant temperature bath and turned on for an interval of 30 min every 3 hr.

The solutions were sampled in the dark and placed in light-tight containers until absorption readings at 550 mmc were made. Results of these series of experiments are shown in Figures 3 and 4 from which it may be seen that light energy is effective in decomposing the brown substances produced upon heating the ascorbic acid. Not only is the rate of browning lower during complete illumination than in the dark, but if a dark reaction mixture is exposed to light there is an immediate decrease in the optical density of the solution.

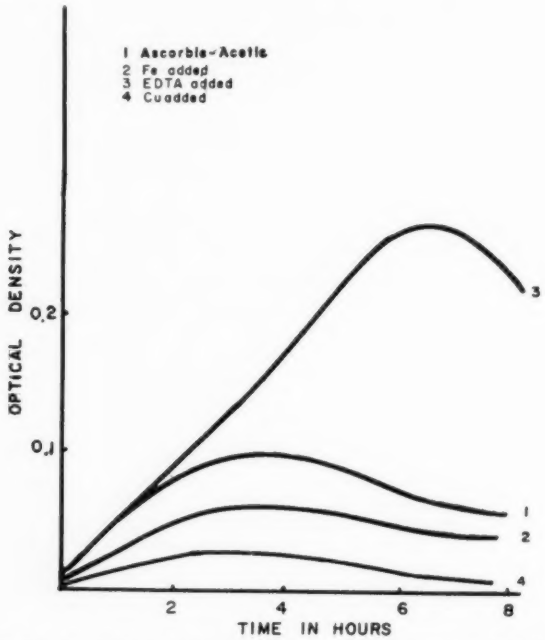

Figure 2. Effect of added substance on the browning of ascorbic-acetic acid solutions.

Contribution of the buffer to carbon dioxide production. Lahikainen *et al (7)* showed that the carbon dioxide evolved during browning of the systems of ascorbic acid, glycine and citrate was not produced by decarboxylation of the glycine. She reported that total carbon dioxide was in excess of that required by the mono-decarboxylation of ascorbic acid. Earlier, Lamden and Harris *(8)* found in solutions of ascorbic acid and citric acid that there was no loss or break-down of citric acids during the browning of the mixture when heated in closed tubes at 100° C. They found also that the quantity of carbon dioxide evolved from a 2.5% ascorbic acid, 50% citric acid solution in 5 hr at 60° C was only 13.5% of the destroyed ascorbic acid.

To determine whether or not the buffer might contribute to the CO_2 formation during browning at low concentrations of reactants, experiments were performed using 1-C^{14} acetate as the buffer.[b] The reaction solution was as before (0.025 molar in ascorbic acid

[b] The 1-C^{14} acetate was prepared from barium carbonate and by the method of Calvin *et al (2)*.

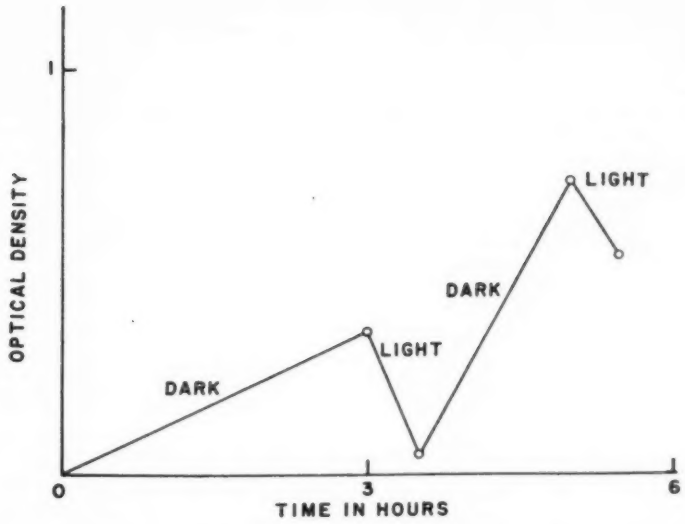

Figure 3. The effect of alternating light and dark periods upon the browning of ascorbic acid solutions.

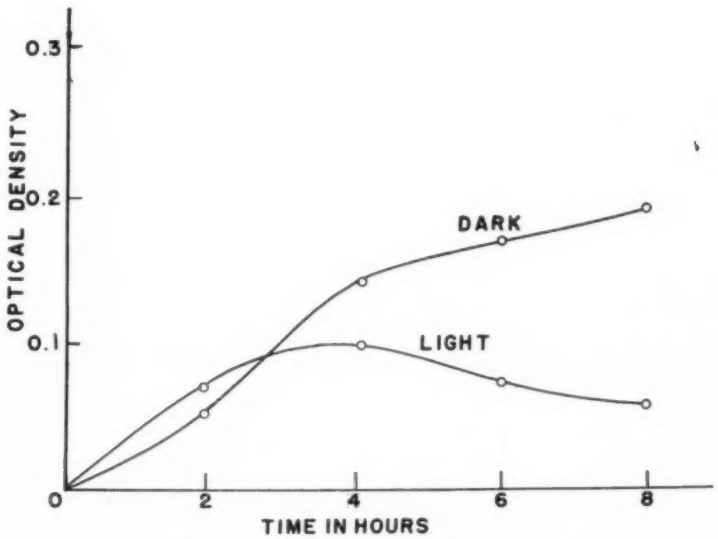

Figure 4. Darkening of ascorbic acid acetate mixture in the presence and absence of light.

and 0.11 molar in acetate buffer). The specific activity of the acetate buffer was 889 counts per mg barium carbonate per min. The solution was incubated at 50° C for 24 hr during which time samples from the CO_2 absorption solution were obtained at 2, 4, 6, 8, and 24 hr. The total quantity of CO_2 at each interval was determined by titration of the CO_2 trapping solution with 0.1 *N* HCl after the addition of barium chloride. The precipitated barium carbonate was assayed for activity in a Tracer-lab gas flow counter. Results are given in Table 1. In all cases less than 4% of the evolved CO_2 was derivable from the acetate buffer.

TABLE 1

$C^{14}O_2$ Production from ascorbic acid-sodium acetate mixtures during browning

Sample time in hours	mg $BaCO_3$	Average count cts/mg/min	% acetate carbon in CO_2
2	135.9	32.07	3.6
4	161.5	28.77	3.23
6	216.7	32.20	3.61
8	246.2	24.62	2.78
24	291.6	20.58	2.31

Ascorbic acid changes. Herbert *et al (4)* presented the absorption spectra of ascorbic acid in the ultra-violet which is characterized by a peak absorption at 270 mmc. By following the changes in absorption at 270 mmc during the initial browning it is possible to determine roughly the disappearance of ascorbic acid. In the experiment with iron or copper, it was noted that the ascorbic acid disappeared at a much more rapid rate in these solutions than in the solutions with no metallic ions. When dehydroascorbic acid was substituted mole for mole for ascorbic acid, in the solutions previously described, the rate of browning was substantially increased.

The reported spectra of dehydro-ascorbic acid showed an anomolous spectra in the 270 to 290 mmc region *(4)*. The diketo gulonic acid, however, displayed a maximum at 290 mmc. When a solution of ascorbic acid was allowed to stand, the absorption at 270 mmc decreased while the absorption at 290 increased. Such a solution, which contained no buffer and had not darkened, was added to an appropriate buffer when the absorption at 290 mmc was maximized. Browning was allowed to proceed at 50° C. In this case browning occurred at a rate approximately equivalent to that found with experiments using dehydroascorbic acid.

In all the experiments upon which complete spectral absorption measurements were made, a characteristic absorption band was noted at 290 millimicrons during browning.

DISCUSSION

During the series of experiments outlined, absorption measurements made from 750 mmc to below 250 mmc with a Beckman DK-2 recording spectrophotometer. As has been reported previously, the darkening or browning of these solutions is caused by an increase in absorption in the 400 to 550 mmc band of the visible spectra and thus most measurements of browning have been made in the 425 to 500 mmc region. During browning, the absorption increases very rapidly in the lower wave lengths and in the majority of cases involving an appreciable amount of browning, the solutions must be diluted in the latter stages of the experiment in order to make a measurement in the 450 mmc region. When such a procedure was attempted for the solutions described here, it was found that dilution invariably decreased the optical density in excess of that expected by the addition of the diluting agent. Since the diluted solutions did not obey Beers law, measurements were made at 550 mmc to avoid the difficulty attendant in the dilution.

Metallic ions in the presence of oxygen increase the conversion of ascorbic acid to dehydroascorbic acid and to diketogulonic acid. If the formation of these compounds were the sole limiting reactions in the browning of ascorbic acid, their accelerated formation by metallic contamination would accelerate the rate of browning. Such is not the case, however, and in fact the experiments outlined showed a negative catalysis. On the other hand, the rate of browning in ascorbic acid buffer systems devoid of metallic ions is increased when the available ascorbic acid is converted to dehydro ascorbic acid. These results would indicate that in a non-metallic system one of the first reactions in the presence of oxygen is a conversion of the ascorbic acid to its dehydro form and may be partially limiting. In the presence of metals, however, a reaction leading to the formation of polymeric compounds must be sensitive to metallic ions and becomes the rate limiting reaction.

The polymerized product initially formed in an ascorbic acid browning system is bleached by ultra-violet light, and the rate of browning is slower in the presence of light than in its absence. In addition to this, as pointed out above, the brown polymeric substances do not follow Beer's law when diluted. It would thus seem that at least a portion of the material responsible for the darkening is of a nature that it is depolymerized by the action of extremely mild agents.

The nature of the intermediates beyond diketo gulonic acid suggested by Hodges would chelate metallic ions, and if this were the case the ability of these materials to form long chains capable of light absorption would be deleteriously effected.

Although the initial products of the browning of ascorbic acid are extremely weakly linked and are disruptable by the action of mild agents the polymerization or browning products formed later are not. The optical density of the systems described rises with time until a maximum is reached; a decrease in optical density then follows, which in turn is followed by a second rise in optical density to a point higher than the original maximum. In the second period of browning the light absorbing material is not bleached by the action of light.

The evolution of CO_2 by browning mixtures of ascorbic acid and buffers at low concentrations is primarily due to the multiple decarboxylation of ascorbic acid.

SUMMARY

The browning reaction of ascorbic acid in a buffer of pH 7 aerated with oxygen forms intermediates which are light-sensitive and sensitive also to the presence of heavy metals. Both of these agents decrease the rate of browning and the first maxima of light absorption reached when the solutions are incubated at elevated temperatures.

In systems devoid of heavy metals, dehydro ascorbic acid or diketo gulonic acid may be substituted for ascorbic acid with a slightly increased rate of browning.

The CO_2 evolved in the darkening of ascorbic acid in oxygen at pH 7 is derived primarily from the ascorbic acid. More than one carbon of the ascorbic acid is lost per unit ascorbic acid converted to other products. Less than 4% of the CO_2 arises from the buffer.

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SOME AROMAS PRODUCED BY SIMPLE AMINO ACID, SUGAR REACTIONS[a,b]

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The color produced by the various nonenzymatic browning reactions, particularly carbonyl-amine reactions, has received considerable attention. Extensive review articles by Danehy and Pigman *(2)* and Hodge *(3)* are good examples of the emphasis placed upon these reactions by various research institutions.

Flavor production is also an important attribute of nonenzymatic browning reactions; yet this feature is often over-looked. Only three direct references to the flavor of carbonyl-amine reactions could be found. Lintner *(4)* reported that the odor and taste of reactions between amino acids and sugars varied with the amino acid. This is an important point, since the flavor or off-flavor resulting from such reactions in a particular processed food may then be related in part to the quantity and quality of reactive nitrogen (amino acids) present in the raw material.

Ruckdeschel *(5)* reported that glycocoll, when reacted with glucose, resembled beer; and that phenylalanine and leucine resembled the odor of dead roses and fresh bread, respectively. Barnes and Kaufman *(1)* found that amino-butyric acid and other amino acids develop the flavor of maple syrup when reacted with glucose.

This report describes the subjective evaluation of flavors produced when a number of amino acids are reacted with glucose at a moderate and at a relatively high temperature. The object was to establish at least one set of flavor impressions obtained by these reactions to suggest how flavor is altered, unbalanced, or may even be improved in certain food processing operations.

METHODS

Browning reactions studied were conducted in the following manner:

Moderate reaction temperature (100° C)

To a test tube was added 50 mg reagent grade glucose, an equimolar amount reagent grade amino acid, and 0.5 ml water. After 1 hr in a boiling water bath, the color intensity was noted, and the sample submitted to a panel for flavor evaluation.

High reaction temperature (180° C)

The reactants, and water in the proportion just described, were added to a 100 ml 3-neck flask. In addition, 10 ml mineral oil (Squibb) was added. After fitting the flask

[a] A research project submitted by the senior author in partial fulfillment of requirements for the degree of B.S. (Food Technology), Cornell University, 1959. The problem was suggested and directed by the Junior author.

[b] Journal Paper No. 1177, New York State Agricultural Experiment Station, Cornell University, Geneva, New York, 1959.

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with a reflux condenser and thermometer, the reaction mixture was heated to 180° C, and then allowed to cool. The oil, which served to retain the volatile reaction products, was then submitted to the panel for odor evaluation. The temperature at which color formation was first observed was recorded for this series of reactions.

Flavor panel

The flavor panel, 6 persons, were usually assembled as 3 groups of 2 persons, or 2 groups of 3 persons. The base flavor and aroma of the reaction mixtures was discussed by these groups, and an attempt was made to describe the base flavor and aroma. The comment reported is that generally agreed upon by the majority of the panel.

RESULTS AND DISCUSSION

Results obtained by reacting amino acids and glucose at the two temperatures are summarized in Table 1; only the most characteristic aromas are treated. As expected, a variety of flavors were produced. The flavor produced in a few instances occurred through amino acid decomposition alone. Cystine and cysteine yield sulfide-like aromas alone, methionine a potato-like aroma, and leucine, a chocolate-like aroma. For the most part, the aromas produced resulted from the reaction between the amino acid and sugar as contrasted with the caramel flavor produced when sugar is caramelized (Table 1).

In addition to the compounds studied at 100° C, but which gave only a caramel flavor when reacted with glucose were glycine, α-alanine, glutamic acid, β-alanine, urea, and ammonium chloride. With few exceptions, the base flavor of the reaction products was bitter; exceptions were alanine (sweet), methionine (potato), and histidine (acid-bitter).

A contrasting set of aroma impressions was obtained when amino acids were reacted with glucose at 180° C. Some amino acids (proline, histidine, lysine), which gave no aroma or gave objectionable aromas at 100° C, yielded pleasant aromas at 180° C. Other amino acids (leucine, threonine, arginine) gave pleasant aromas at 100° C, and objectionable aromas at 180° C. Still other amino acids (valine, glutamine, aspartic acid) yielded

TABLE 1

Some aromas produced upon reacting amino acids with glucose in simple two-component systems at a moderate and at a relatively high temperature

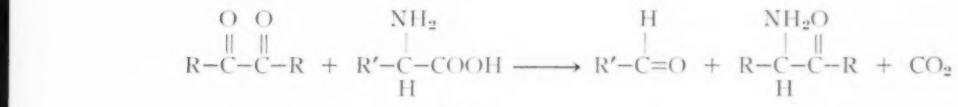
Amino acid	100° C		180° C	
	Aroma	Color*	Aroma	Temp. of initial browning ° C
None (glucose only)	None	0	Caramel	150
Valine	Rye bread	X	Penetrating, chocolate	80
Leucine	Sweet, chocolate	0	Burnt cheese, objectionable	120
Isoleucine	Musty	XX	Burnt cheese, objectionable	110
Proline	Burnt protein	XX	Pleasant, bakery aroma	120
Methionine	Potato	X	Potato	110
Glutamine	Chocolate	XX	Butterscotch	130
Threonine	Chocolate	XX	Burnt	100
Histidine	None	XX	Corn bread	120
Aspartic	Rock candy	0	Caramel	60
Arginine	Popcorn	XXXX	Burnt sugar	60
Lysine (hydrochloride)	None	XXX	Bread-like	130
Phenylalanine	Violets	X	Violets, lilac	80

* 0 denotes no color, XXXX denotes very dark brown.

pleasant, but distinctly different, odor characteristics at the two temperatures studied. At 180° C, glycine, alanine, tryptophane, tyrosine, arginine, asparagine, glutamic acid, serine, α-aminobutyric acid, urea, and ammonium chloride gave odors that could only be described as caramel, or burnt sugar. The base flavor of the high temperature reaction products was invariably bitter.

As measured by color intensity, or the temperature at which the reactions studied were initiated (Table 1), considerable difference was found in the rate and apparent completeness of the various amino acid, sugar reactions. The extent to which the reactivity or relative completeness of the reactions affected the flavors observed cannot be assessed at present. Briefly, differences in observed reaction rates correspond with the findings of others *(1, 2)*.

Mechanism for flavor production. One source of aroma in sugar, amino acid reactions is undoubtedly the production of aldehydes through Strecker degredation of the amino acid *(1, 3)*.



The aldehyde produced by Strecker degredation corresponds to the carbon chain of the amino acid minus one carbon atom lost through decarboxylation. This reaction serves to explain why the aroma produced by certain sources of reactive nitrogen can only be described as caramel. For example, ammonium chloride would not produce an aldehyde; the acetaldehyde of alanine is not particularly odoriferous and being highly reactive, may polmerize immediately upon being formed. The aldehyde produced by tyrosine would not be expected to be volatile *(1)*.

Mechanisms responsible for the production of volatile flavor other than Strecker degredation must be operative to explain the changing odor quality found when the reaction temperature is altered. Barnes and Kaufman *(1)* found that the flavor of baked soy flour varied with the time, temperature, moisture content, and pH of the baking operation. Without question, multicomponent model amino acid, sugar reaction systems would yield a great variety of flavors. Chemical and organoleptic evaluation of such reactions should yield information pertinent to the mechanism of color and flavor production in a variety of processed foods.

SUMMARY

A variety of flavors, particularly aromatic, was observed when amino acids were reacted with glucose in simple, two-component model systems. The various flavors produced, and the changing flavor character observed when temperature was altered may suggest where flavor may be unbalanced, altered, or improved in food processing operations.

Acknowledgments

The authors are indebted to Dr. E. A. Nebesky for the interest he took in this study as the program advisor of the senior author. The majority of the work was accomplished using laboratory space kindly provided by Dr. B. L. Herrington.

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CARBONYLS IN OXIDIZING FAT
III. THE DISTRIBUTION OF VOLATILE AND NON-VOLATILE CARBONYLS[a]

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The determination of carbonyls is useful in the study of flavor deterioration in fat containing foods. Reported methods *(4, 6, 15, 22, 28, 29)* have usually involved isolation by some type of volatilization procedure and estimation as 2,4-dinitrophenylhydrazones.[b] Steam volatile carbonyl 2,4-DNPH's have been separated into mono- and dicarbonyl fractions *(15)*, and the monocarbonyls resolved into classes *(14, 15)* and individual compounds identified *(13, 14, 16, 29)*. The carbonyls in the fat may be converted to 2,4-DNPH's, but complete separation from the fat has proven difficult. Procedures for the estimation of free monocarbonyls *(30)*, and total determinable carbonyls and saturated and unsaturated components thereof *(19)*, in the presence of the fat have been devised.

The carbonyl components in oxidized fat are extremely complex. The greater part of the determinable carbonyls are non-volatile *(2, 8, 20, 22, 27)* and are of unknown structure. Lea and Swobodi *(22)* have shown that aldehydes of C_6–C_{14} chain length can be recovered practically quantitatively from fats by vacuum distillation at 50° C (122° F). However, this procedure applied *(22)* to autoxidized fat yielded only 3% of the total carbonyls as determined by the method of Henick, Benca, and Mitchell *(19)*. McKerrigan *(27)* and Chipault, Privett, Mizuno, Nickell, and Lundberg *(8)* have indicated that the volatile carbonyls may be mainly associated with "off" flavors. Holm, Ekbom, and Wode *(20)* and McKerrigan *(27)* have expressed the belief that the non-volatile carbonyls are of high molecular weight and are precursors of volatile odorous substances.

This report describes comparisons of various methods of isolating and estimating carbonyl compounds in autoxidized pork fat. The purpose of this work was to investigate the nature of the non-volatile carbonyls, and obtain information useful in the selection of procedures best suited to the study of relationships of carbonyls with rancid flavors.

The Girard T reagent *(17)* was used for the isolation of the non-volatile carbonyls from the fat. This reagent forms water soluble chlorotrimethylammonium acetylhydrazones with a wide variety of carbonyls. Its use has been recommended by several investigators *(11, 33, 35)* but little has been reported on its application to autoxidized fats. Hammond and Bird *(18)* have recently described its use in the study of autoxidized butterfat.

Solvents, reagents, materials, and equipment. These items were the same as used in similar operations described in earlier papers *(10, 13, 14,*

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[b] Hereafter referred to as 2,4-DNPH's.

15, 16) or in the particular method cited. Methanol and ethanol were purified by refluxing over sodium hydroxide and aluminum and distilling.

Vacuum distillation *(22)* of the fat was carried out in a 241.3 mm-long, flat bottomed tube of 41.3 mm inside diameter with a side-arm 177.8 mm from the bottom. The condensing surface, or cold finger, was a flat bottomed tube with 31.8 mm outside diameter. This was held in place at a depth of 206.4 mm in the larger tube (open end ground) by a bored large rubber stopper, which was placed in an inverted position on it. The end of the dry ice-cooled finger was 20.6 mm from the surface of a 10.0 g-sample of fat. A magnetic stirrer was used to agitate the fat.

EXPERIMENTAL

Fat used in this work was lard oxidized under ultraviolet light at 38° F (3.33° C) and 70° F (21.1° C) and in an oven at 60° C (140° F). As in previous studies *(13, 15, 16)*, determinations were made on unheated fat and heated fat. The fat was heated at 165° C (329° F) for 15 min to approximate the effect of cooking.

Free carbonyls were determined by several procedures. Volatile carbonyls were determined by vacuum distillation of the fat at 50° C (122° F) for 1 hr at 1.0–0.5 mm pressure. Carbonyls condensed on the cold finger were reacted with 150 ml 2,4-dinitrophenylhydrazine reagent,[c] and the hydrazones isolated *(15)*. Steam volatile carbonyls were isolated as 2,4-DNPH's as described by Gaddis, Ellis, and Currie *(15)*. Free monocarbonyls in the fat were determined by the method of Pool and Klose *(30)*. This method was extended to the approximation of free dicarbonyl and dicarbonyl-like compounds by thorough elution of the alumina column with ethanol. Total determinable carbonyls were estimated by the method of Henick, Benca, and Mitchell *(19)*. Total carbonyls were isolated with the Girard T reagent *(17)*. Ten grams fat were treated with 20 ml ethanol containing 5% Girard T reagent. Reactions were run by refluxing 1 hr with or without 0.2 g Dowex 50 cation resin *(35)*.[d] They were also run by shaking at room temperature in an N_2 atmosphere for 3 hr. The Girard reaction mixtures were treated with 200 ml water and the fat recovered by extraction with petroleum ether. The water soluble Girard hydrazones were worked up by two different methods.

Method 1. The Girard hydrazones were treated with 220 ml 2,4-DNP reagent *(15)*. After standing overnight at room temperature, the 2,4-DNPH's were extracted with CCl_4 *(15)*. A second much smaller fraction was obtained by warming the extracted reaction mixture 1 hr on the steam bath and extracting again. CCl_4 extracts were combined.

Method 2. The aqueous solution of Girard hydrazones was made ½ *N* with concentrated HCl and steam distilled 22 min into 2,4-DNP reagent *(15)*. This, after standing overnight, yielded the volatile carbonyl hydrazones. The residue from the steam distillation was treated with 50 ml 2,4-DNP reagent, and the next day the non-volatile 2,4-DNPH's were extracted with CCl_4.

Fat hydroperoxides were reduced with sodium bisulfite by the method of Knight and Swern *(21)*. Aqueous solutions of carbonyl bisulfites were converted to 2,4-DNPH's by addition of 4 ml concentrated HCl per gram of sodium bisulfite *(32)* and an excess of 2,4-DNP reagent.

Separations of total 2,4-DNPH's into mono- and dicarbonyls, and the monocarbonyls into classes, were made according to previously described methods *(13, 14, 15, 16)*.

All determinations or reactions were run on 10 g fat except where the method specified smaller amounts. Quantitative figures were based on absorbance values at the wavelength of maximum absorption of the carbonyl hydrazones from 10 g fat in 100 ml of solution. Spectrophotometric readings were run in CCl_4 except in the Henick *(19)* method.

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[c] Hereafter referred to as 2,4-DNP reagent.

[d] The mention of specific trade names throughout this paper does not constitute endorsement of the product used over comparable materials.

RESULTS AND DISCUSSION

Volatile and free carbonyls. Differences between comparable methods were due to the degree of decomposition of hydroperoxides *(9, 23)* and/or complex secondary oxidation products *(6, 7)*. A comparison of vacuum distillation, steam distillation, and Pool and Klose values is shown in Figure 1 for lard oxidized under ultraviolet light at 38° F (3.33° C) and 70° F

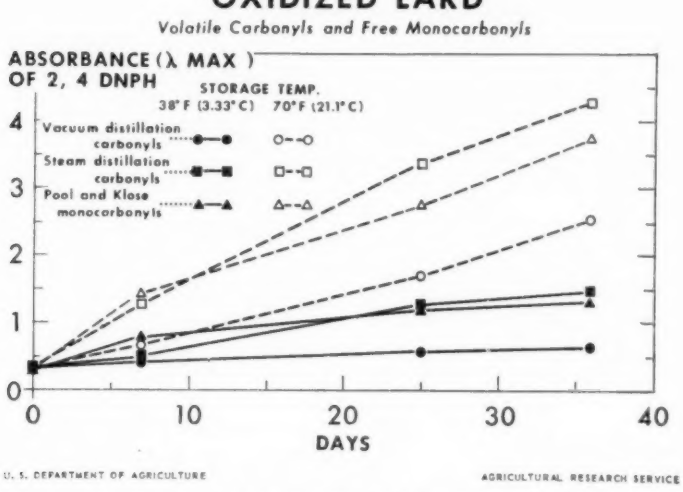

Figure 1. Oxidized lard. Volatile carbonyls and free monocarbonyls.

(21.1° C). Steam distillation values were higher than those of vacuum distillation. Some loss of C_1–C_5 carbon chain monocarbonyls *(22)* could occur in the vacuum distillation, but this was probably of little significance since it has been shown *(16)* that very small proportions of such compounds are present in steam distillates. The differences between steam and vacuum distillation were evidently due to breakdown of peroxides or other precursors. Pool and Klose values were generally lower than the steam distillation values, but the former are free (volatile and non-volatile) monocarbonyls. Vacuum and steam distillation values averaged 2.5% and 4.0% of the Henick value for total carbonyls.

Volatile carbonyl 2,4-DNPH's were separated into mono- and dicarbonyls on 20% hydrated alumina. Proportions of the dicarbonyl fraction were approximately the same in the two methods. Figure 2 is a comparison of monocarbonyls found in unheated and heated lard oxidized under ultraviolet light at 38° F (3.33° C). Pool and Klose values were the highest. The difference between these and vacuum distillation monocarbonyls should represent free non-volatile monocarbonyls, provided that hydroperoxides and other precursors *(3, 31)* are not decomposed on the hydrated alumina. It is interesting that heating [165° C (329° F)] the fat did not decrease the differences between steam and vacuum distillation monocarbonyls. The

temperature of 176° C is a far more severe treatment than steam distillation and should decompose sensitive hydroperoxides rapidly *(9, 23)*. The steam apparently exerted a special effect. Increase in steam volatile monocarbonyls produced by heating is a function of the degree of oxidation *(15)*, and results in large increases in the proportion of unsaturated components *(15, 16)*. The data indicated that the former statement was also true for vacuum distillation and Pool and Klose values. Furthermore, increases in the 3 values as the result of heating were approximately the same. The value for the non-volatile monocarbonyls was not significantly changed by heating. Heating an oxidized lard increased the steam volatile carbonyls 31.5%.

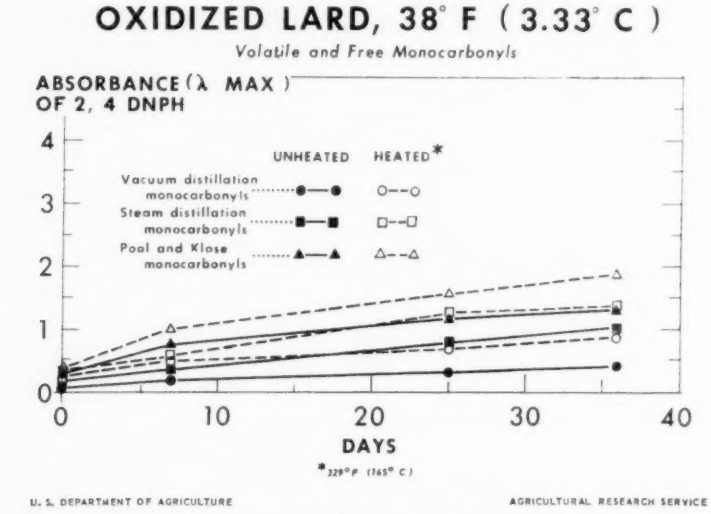

Figure 2. Oxidized lard 38° F (3.33° C). Volatile and free monocarbonyls.

However, steam distillation of the same unheated lard with trichloroacetic acid produced a 63.1% increase. The sum of values from vacuum distillation followed by steam distillation of recovered fat was the same as steam distillation of the whole fat . Pool and Klose, steam, and vacuum distillation monocarbonyls averaged 4.4%, 2.9%, and 1.4%, respectively, of the Henick value for total carbonyls. Shifts in the wave length of maximum absorption of the carbonyl fractions were similar to those described by Gaddis, Ellis, and Currie *(15)*.

Non-volatile and total determinable carbonyls. The Henick method for total determinable carbonyls probably causes extensive decomposition of hydroperoxides as well as complex secondary oxidation products *(20)*. The Girard T reagent offers a means of isolating non-volatile carbonyls. Teitelbaum *(35)* recently reported use of a cation exchange resin instead of acetic acid *(17)* in the reaction. This method was used in the following experiments, with refluxing in ethanol. Products were worked up by reaction with 2,4-DNP reagent and solvent extraction (Method 1). Figures 3 and 4

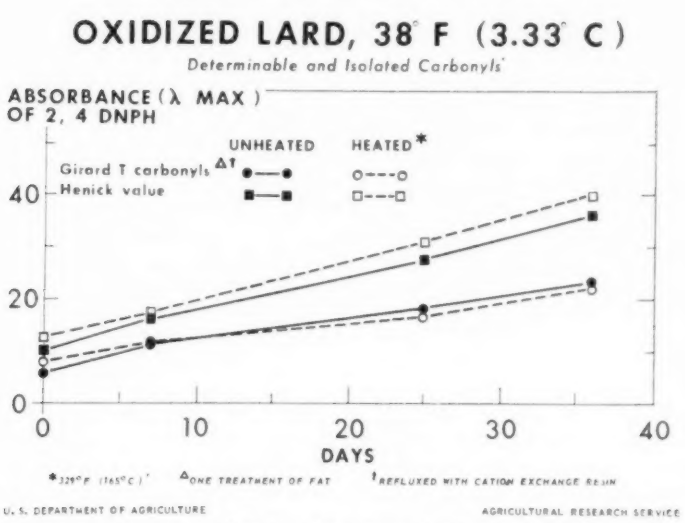

Figure 3. Oxidized lard 38° F (3.33° C). Determinable and isolated carbonyls.

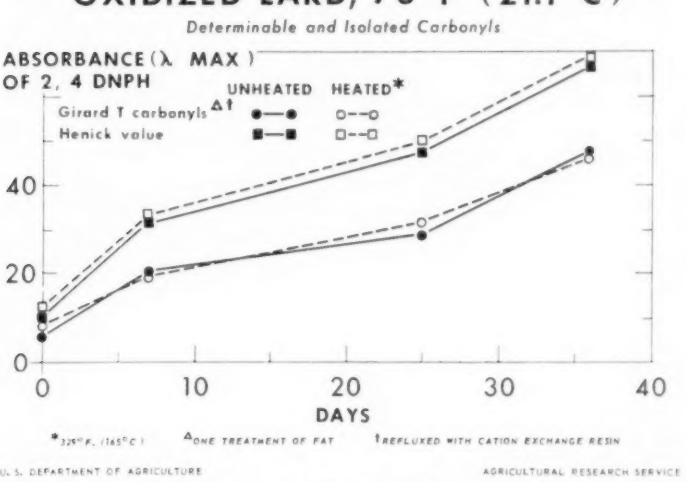

Figure 4. Oxidized lard 70° F (21.1° C). Determinable and isolated carbonyls.

compare Girard and Henick values on unheated and heated lard oxidized at 38° F (3.33° C) and 70° F (21.1° C). Increases in the two values as the result of heating were small and not related to the degree of oxidation. The differences in the Girard values were not significant. This is in contrast to the effect of heating on volatile carbonyls. It indicated that both procedures broke down the same precursors that were decomposed by heat.

Girard T reagent, under these conditions, therefore, isolated both free and formerly bound carbonyls. Girard values averaged 64% of the Henick values. The partial isolation was probably due to the presence of more stable complexes. The Girard reaction removed all free monocarbonyls, as Pool and Klose determinations on recovered fat were negative. The sum of Girard values and Henick values on the recovered fat was approximately equal to Henick values on the whole fat.

Distribution of carbonyls isolated by Girard T reagent. Decomposition of Girard hydrazones by steam distillation in the presence of mineral acid, followed by conversion of the separated volatile and non-volatile carbonyls to 2,4-DNPH's (Method 2), gave an indication of the nature and distribution of the carbonyls isolated. Results are shown in Table 1. The lard used was oxidized at 60° C (140° F) to a peroxide value of 15. Girard derivatives were prepared by refluxing with Dowex 50 cation exchange resin. Results were similar for unheated and heated lard. The data show the presence of 41.1% volatile carbonyls, 33.6% monocarbonyl, 29.7% volatile monocarbonyl, and 3.9% non-volatile monocarbonyl based on total carbonyl isolated. The non-volatile monocarbonyl fraction was therefore of the order indicated by differences between Pool and Klose and vacuum distillation values. Volatile carbonyl fractions isolated by the Girard T reagent, although mostly coming from the breakdown of precursors, were at least superficially similar to the free volatile carbonyls present in the fat. The volatile monocarbonyls were separated by paper chromatography *(14, 15)* into the characteristic saturated aldehyde, 2-enal, and 2,4-dienal classes *(15, 16)*. The non-volatile monocarbonyls separated into just one spot. The maximum of this material was 355-8 mmc, which may represent a new class *(16)*. Dicarbonyls were 66.4% of the total carbonyls isolated. Volatile licarbonyls were 11.4% of the total, had a maximum at 380-5 mmc *(15)*, and were present in the proportions usually found in steam volatile carbonyls *(15)*. Non-volatile dicarbonyls, or dicarbonyl-like compounds, were 55.0% of the total. These gave a red color with alcoholic alkali. An appreciable amount of these could not be removed from the 20% hydrated alumina with ethanol. The proportion of these compounds that are present as free non-volatile dicarbonyls is not known. Further examination of the fraction retained by the alumina in the Pool and Klose separation might provide a partial answer. However, contact with hydrated alumina may be attended by decomposition of oxidation products *(3, 31)*. These non-volatile dicarbonyls are evidently of high molecular weight and could be precursors of the volatile carbonyls.

The Girard reaction. It is evident that much of the carbonyl was released in some manner by the Girard reagent and reaction conditions. The carbonyls may be present in bound forms or arise from oxidative polymers *(6, 7)* or partial decomposition of hydroperoxides. Production of carbonyls is probably influenced by the heat, the cation exchange resin and possibly by the Girard T reagent. It would be useful if the action of the Girard reagent could be limited to free carbonyls.

The literature indicates that cation exchange resins are frequently powerful catalysts. They are known to catalyze esterification, interesterification, and saponification *(25, 34, 36)* and form acetals *(34)*. Cation ex-

TABLE 1

The distribution of carbonyls isolated by the Girard T reagent [1]

Lard oxidized at 60° C (140° F) to peroxide value of 15	Carbonyl 2,4-dinitrophenylhydrazones								
	Absorbance (λ max)	Percent							
		Total		Volatile			Non-volatile		
		Mono-carbonyls	Di-carbonyls	Carbonyls	Mono-carbonyls	Di-carbonyls	Carbonyls	Mono-carbonyls	Di-carbonyls
Unheated lard									
Method 1	29.7	33.3	66.7						
Method 2	24.6	34.9	65.1	41.9	31.2	10.7	58.1	3.7	54.4
Heated lard									
Method 1	30.3	30.4	69.6						
Method 2	30.5	32.3	67.7	40.3	28.2	12.1	59.7	4.1	55.6
Average values									
Method 1		31.9	68.1						
Method 2		33.6	66.4	41.1	29.7	11.4	58.9	3.9	55.0

[1] Refluxed with Dowex 50 cation exchange resin.

TABLE 2

Effect of reaction conditions on yield of Girard carbonyls

Lard oxidized at 70° F (21.1° C)	Total carbonyl 2,4-dinitrophenylhydrazones absorbance (λ max)							
	Repeated treatments of lard							
	Cation exchange resin				No cation exchange resin			
	No. 1	No. 2	No. 3	Total	No. 1	No. 2	No. 3	Total
Refluxed								
Unreduced lard								
Method 1	40.66	12.95	2.34	55.95	65.20	5.37	2.30	72.87
Method 2	32.00	12.30	3.07	47.37	35.80	4.53	2.30	42.63
Reduced lard								
Method 1	22.02	6.64	2.50	31.16	16.16	5.80	5.62	27.58
Method 2	18.60	7.83	1.93	28.36	13.15	5.36	5.47	23.98
Room temperature								
Unreduced lard								
Method 1	62.20	21.83	6.45	90.48	17.44	6.78	2.86	27.08
Method 2	39.00	21.00	6.08	66.08	15.80	6.14	2.54	24.48
Reduced lard								
Method 1	24.75	9.04	.....	33.79	15.72	6.04	6.08	27.84
Method 2	23.60	8.98	.....	32.58	11.42	3.80	5.46	20.68

change resins also are capable of catalyzing condensation and polymerization of aldehydes *(1)*. Much of this potential catalytic action was probably inhibited by the two-phase system, fat and ethanol, in which the reaction was run. The ability of replacing mineral acids makes it seem likely that the chief action was depolymerization of oxidative polymers *(6, 7)* and decomposition of hydroperoxides.

Girard and Sandulesco *(17)* specified the use of acetic acid as catalyst in the reaction. However, Lederer and Nachmias *(24)* have stated that good yield was obtained in the Girard reaction without the use of acid. Hammond and Bird *(18)* reported that the peroxide value of oxidized butterfat was unchanged when the reaction was run without acid or catalyst at 40° C (104° F). The hydroperoxides can be reduced with sodium bisulfite *(21)*. Such a step involves opposing side reactions *(26)*. Carbonyls presumably free, are extracted as bisulfites. These may be accounted for by conversion to 2,4-DNPH's *(32)*. Unsaturated carbonyls may be lost through the formation of sulfonic acid derivatives *(32, 37)*. There may also be an increase in free carbonyls through the reduction of low molecular weight peroxides of the per acid type *(12)*.

Data in Table 2 show the effect of cation exchange resin, heat, and reduction of hydroperoxides on the total carbonyls isolated. The lard used in the experiment was oxidized under ultraviolet light at 70° F (21.1° C) to a peroxide value of 32.0 The Girard reaction was repeated twice in succession on recovered lard. Reaction mixtures were worked up by Methods 1 and 2. Total carbonyls found by the various other methods were vacuum distillation 2.08, steam distillation 4.60, Pool and Klose 3.96, and Henick 70.67. Reduction with sodium bisulfite diminished the peroxide value to 0.4. The fat then had carbonyl values of vacuum distillation 1.65, steam distillation 5.16, Pool and Klose 3.80, and Henick 45.11. Carbonyl bisulfite extracted during the reduction was 5.83. Carbonyl 2,4-DNPH's isolated as bisulfites were 75.6% monocarbonyls. The maximum of this fraction indicated it was composed of saturated aldehydes. The 12.2% dicarbonyls could not be eluted from the hydrated alumina with ethanol. Since free carbonyl values were little changed, the reduction apparently resulted in an over-all increase in free carbonyl despite the potential loss of unsaturated free carbonyls. The Henick value (45.11) for total determinable carbonyls was considerably decreased by reduction. Girard isolated carbonyls were greatly diminished by reduction, except at room temperature without cation exchange resin. This indicated that hydroperoxide decomposition was produced by both cation exchange resin and heat. Results of Method 2 generally correlated with those of Method 1, but were much lower. Apparently steam distillation in the presence of HCl was destructive of carbonyl compounds. Oddly, this effect was greatest in the initial reaction. The combination of heat and cation exchange resin decreased the yield of carbonyls. However, at room temperature the presence of cation resin gave the highest total values. The lowest yield of carbonyl from unreduced lard was at room temperature without catalyst. Both heat and the cation exchange resin in the presence of Girard T reagent were able to break down hydroperoxides and other precursors. The cation exchange resin was probably more effective than heat in decomposing the latter.

TABLE 3

Influence of reaction conditions on distribution of Girard isolated carbonyls [1, 2]

	Carbonyl 2,4-dinitrophenylhydrazones absorbance (λ max)									Percent		
	Total	Steam volatile	Non-volatile	Total		Volatile		Non-volatile		Mono-carbonyl	Non-volatile dicarbonyl	Dicarbonyl retained by alumina
				Mono	Di	Mono	Di	Mono	Di			
Cation exchange resin												
Refluxed												
Method 1	40.66			12.30	16.92					30.2		28.2
Method 2	32.00	17.68	14.32	14.51	8.84	13.33	2.12	1.18	6.72	45.3	41.1	27.1
Room temperature												
Method 1	62.20			15.76	24.00					25.3		36.1
Method 2	39.00	20.80	18.20	13.68	11.26	13.22	2.78	0.39	8.48	35.0	45.7	36.1
No cation resin												
Refluxed												
Method 1	65.2			29.30	21.40					44.9		22.2
Method 2	35.8	21.4	14.4	21.05	7.59	18.80	2.30	2.25	5.29	58.7	33.3	20.0
Room temperature												
Method 1	17.44			4.57	8.34					26.2		26.0
Method 2	15.80	6.26	9.54	5.40	6.20	4.63	0.95	0.77	5.25	34.2	55.5	26.6

[1] From first treatment of fat with Girard T.
[2] Unheated unreduced lard.

TABLE 4

Carbonyl distribution analysis of an oxidized lard [1]

Girard reaction [2]	2, 4-Dinitrophenylhydrazones								
	Lard					Reduced lard			
	Absorbance (λ max)				Percent Mono-carbonyls	Absorbance (λ max)			Percent Mono-carbonyls
	Total carbonyls	Mono-carbonyls	Di-carbonyls	Henickon recovered lard		Total	Mono-carbonyls	Di-carbonyls	
Refluxed, cation resin	17.64	6.86	7.12	9.26	39.0	13.20	3.36	5.70	25.4
Refluxed, no cation resin	20.00	6.98	9.39		34.6	11.52	2.48	5.48	21.5
Room temperature, cation resin	25.08	7.52	11.40	11.70	30.0	16.88	4.11	6.76	24.3
Room temperature, no cation resin	13.10	3.06	6.72		23.4	15.44	3.12	6.70	20.2
Henick value	35.74					28.65			
Pool and Klose value		2.30	3.95						
Steam distillation	2.00	1.25	0.38		62.5	3.36	2.06	0.44	61.0
Bisulfite extracted	0.95	0.63	0.19		66.3				

[1] Peroxide = 18.
[2] Initial reaction with Girard A reagent.

The Girard reagent, even under the mildest of conditions, appeared capable of breaking down precursors. This was indicated by the isolation of more carbonyl on repeated treatment. The initial value for lard at room temperature without catalyst could approximate the free carbonyls present. The Pool and Klose monocarbonyl value for unreduced lard was 3.96. Elution of the 20% hydrated alumina column with ethanol gave a dicarbonyl 2,4-DNPH fraction of 7.53. This gave a total carbonyl of 11.49. However, ethanol did not completely remove the 2,4-DNPH's from the column. When the lard in benzene solution was applied to a 20% hydrated alumina column, and the column eluted with benzene and then ethanol, the resulting fractions, when tested with 2,4-DNP reagent, had values of 5.29 and 11.80. The total, 17.09, agree closely with the initial Girard value, 17.44. Thus it may be possible to isolate only free carbonyls with the Girard T reagent by the use of optimum conditions.

Table 3 is an extension of the data in Table 2, showing the distribution of carbonyls isolated in the initial Girard reaction on the unreduced lard. The loss of carbonyls by Method 2 is clearly shown to be in the dicarbonyl fraction. Amounts of the various fractions were influenced by the reaction conditions. Approximately 25-30% of the total carbonyls could not be removed from the hydrated alumina with ethanol. Further study of the Girard reaction with autoxidized fat is required. The complexity of the reaction is emphasized by the comprehensive data in Table 4 for a lard oxidized under UV light at 70° F (21.1° C) to a peroxide value of 18. This analysis generally supports the conclusions already stated. Yields of carbonyls were diminished in both reduced and unreduced lard by the combination of the cation exchange resin and heat, and heat alone. Most of this effect appeared to be on the dicarbonyl fraction. Production of carbonyl through hydroperoxide breakdown was an important source of differences. Apparently, hydroperoxide decomposition was minimized at room temperature without cation resin. Values obtained under mild conditions may then approach the actual free carbonyl content, which is much lower than the total carbonyl determinable.

SUMMARY

A study has been made of the distribution of volatile and non-volatile carbonyls in oxidized lard. Most of the carbonyls determinable were non-volatile and/or bound. Results indicated that a large part of the determinable carbonyls do not originally exist in the oxidized fat as carbonyls. They apparently are produced through breakdown of precursors by reaction conditions. Free monocarbonyls were 4.4%, volatile monocarbonyls 1.4%, and volatile dicarbonyls 1.1% of the total determinable carbonyls. Initial reaction with the Girard T reagent isolated an average of 64% of the total carbonyls determinable. In a moderately oxidized fat, carbonyls isolated by the Girard reagent were 33.6% monocarbonyl and 66.4% dicarbonyl, 41.1% volatile carbonyl and 58.9% non-volatile carbonyl, 29.7% volatile monocarbonyl and 11.4% volatile dicarbonyl, and 3.9% non-volatile monocarbonyl and 55.0% non-volatile dicarbonyl. The amount of carbonyls isolated by the Girard reagent was greatly influenced by reaction conditions. In general, results emphasize the need for a reliable method of isolating and determining the free total carbonyl in fat.

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BIOSYNTHESIS OF ANTHOCYANINS IN RADISHES

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In general, the anthocyanins are one group of the flavonoid compounds possessing a structure based upon a C-6, C-3, C-6 carbon skeleton (Fig. 1). Although the physiological role of these pigments in plants is not known, it is evident that they are produced in plants from photosynthetically fixed carbon sources. Robinson *(8)* postulated that the anthocyanins are formed from simple carbohydrates and speculated that the C-6, C-3, C-6 system is built from a pair of hexoses and a triose by means of an aldol condensation.

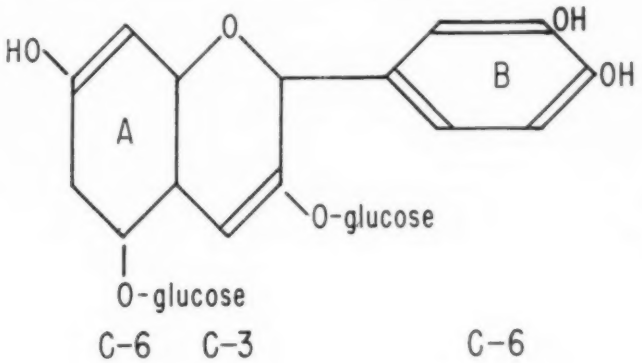

Figure 1. Cyanidin diglucoside.

Birch, Donovan and Moewus *(1)* studied the formation of quercetin in *Chlamydomonas eugametos,* and found that the C-3, C-6 moiety of quercetin was derived from compounds resembling phenylalanine. They also suggested that ring A originated from phloroglucinol. Collie *(5)* earlier had suggested that the head to tail linkage of acetic acid might be involved in the biosynthesis of phenolic compounds. Birch *(2, 3)*, using isotopically labeled acetic acid, showed that the phloroglucinol portion of cyanidin was derived from acetic acid. The carbon from carboxyl-labeled acetic acid contributed the ring carbons in the phloroglucinol which carried the oxygen atoms. When methyl-labeled acetate was used two-thirds of the labeling was found on the carbons between the hydroxyl groups.

Other investigators *(7, 9, 10)*, using *Escherichia coli* and *Aerobacter aerogenes* mutants, showed that shikimic acid could replace phenylalanine in the formation of prephenic acid and phenyl pyruvic acid. It was also shown that shikimic acid is derivable from the pentose pathway.

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In the synthesis of quercetin Neish *(11, 12)* has shown that ring A arises from the head to tail linkage of acetic acid and ring B from sugars via shikimic acid and phenylalanine.

In this paper we report upon the formation of the diglucoside of cyanidin in radish subepidermal tissue.

EXPERIMENTAL

A. Plant material. Commercial varieties of radish (Raphanus sativus) were grown in a field house under normal conditions. The radishes used were freshly harvested, and were usually 4–6 weeks old.

B. Incorporation of radio-active compounds. The leaves and tips of the radishes were cut off and the remainder washed with water and wiped. The surface was gently rubbed with a course test tube brush to promote the infiltration of radio-active compounds.

Solutions of uniformly labeled radio-active sugars[b] and carboxyl labeled acetate[c] were smeared on the rubbed surface of the radish with a rubber tip of a stirrer rod. The radishes were then put in covered dishes and were left for 24 hr at room temperature in the light.

C. Determination of radio-activity. The starting materials as well as the isolated compounds were counted on chromatographic paper. The total number of counts were recorded and the background counts of an equivalent area of paper subtracted to give the net counts of the active material.

D. Isolation of anthocyanin. 1. *Extraction.* Twenty-four hours after the incorporation of radio-active compounds the radish tubers were peeled and the peelings macerated in a mortar. These were extracted exhaustively with absolute methanol until all the color was removed. The methanol extract was concentrated under vacuum. Losses during concentration were 1–5% as measured by losses in total activity. The volumes of the concentrates were measured and their activity determined after spotting on Whatman No. 1 filter paper.

2. *Separation of the radish anthocyanin by paper chromatography.* Due to the small quantities of anthocyanin used in the experiments paper chromatographic separations were used to isolate the compound from the other water soluble materials in the radish tuber. The extract of the radishes was chromatographed using a solvent composed of butanol, acetic acid, water saturated with picric acid (4:1:5 v/v). The use of picric acid saturated water in the solvent decreased the trailing of the separated anthocyanins markedly. All chromatograms of pigments were developed by the use of an ascending technique in a single solvent. The bands were then cut from the paper, eluted, and reseparated twice.

After separation, the anthocyanin was counted to determine its total activity.

E. Hydrolysis of anthocyanin. 1. *Isolation of glucose after the determination of radio-activity.* The anthocyanin spots on the picric acid saturated paper were cut out and eluted with 50% methanol. The eluate was concentrated under vacuum to 0.1 to 0.2 ml and extracted repeatedly with ether to eliminate the picric acid. An equal volume of concentrated hydrochloric acid was added and the mixture heated on a steam bath for 3 min. The hydrolysate was passed through a short column of Dowex-1 to eliminate the colored compounds and the remaining picric acid. The eluate was then successively passed through columns of Amberlite IR-120 and Amberlite IRA-400 to eliminate other ionic materials. The eluate, primarily glucose, was then concentrated and chromatographed using butanol, acetic acid, water (4:1:5 v/v). The glucose was identified by spraying with an indicator consisting of 0.2% phenylenediamine and 2% oxalic acid in ethanol. The total radio-activity of the sugar was then determined by counting the entire indicated area on the paper.

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[b] Prepared by the infiltration of $C^{14}O_2$ into tobacco leaves, and the separation of the alcohol solubles by paper chromatography *(6)*.

[c] Prepared from $C^{14}O_2$ and CH_3MgI by the method of Calvin *et al (4)*.

2. Preparation of cyanidin chloride. A separate larger aliquot of the extract was hydrolyzed as above. After cooling, it was centrifuged to remove the crystals of cyanidin chloride which were repeatedly washed with one per cent hydrochloric acid until the aqueous phase became light in color. The precipitate was then dried under vacuum and weighed. A portion was dissolved in absolute ethanol and spotted on paper for the determination of activity.

F. Degradation of cyanidin chloride. 1. *Alkaline fusion.* A modified method of fusion was developed for use on a microscale. Ten milligrams of cyanidin chloride were dissolved in 1 ml of absolute ethanol. This was added to 0.48 g of potassium hydroxide and 0.18 ml water in a platinum crucible. The mixture was placed in an oven at 250° C for 5 min. The platinum crucible was then floated on water and 2 or 3 ml of water were added to the fusion products. Concentrated hydrochloric acid was added until the mixture was acid. The products were then extracted with about 100 ml ether to recover the phloroglucinol and protocatechuic acid.

2. Isolation of phloroglucinol and protocatechnic acid. Ether extracts of the alkaline fusion products were streaked on two papers of 15 x 45 cm in size. A chromatogram was run with the solvent used previously. After separation, the edges of the paper were removed for development with diazotized paranitroaniline. The bands of protocatechuic acid and phloroglucinol so indicated were cut from the paper and eluted with absolute ethanol. The two eluates were concentrated and rechromographed in the same solvent. Two portions of the paper were sprayed with diazotized paranitroanaline and used subsequently for the determination of radio-activity. The remainder of the bands were eluted from the paper with absolute ethanol for the colorimetric determination of the protocatechuic acid and phloroglucinol.

3. Colorimetric determination of protocatechuic acid and phloroglucinol. a. Protocatechuic acid. The protocatechuic acid eluted from the paper was reacted with ferric chloride to give a green color. The optical density was determined at 450 mmc with a Beckman DU spectrophotometer and the concentration obtained from the standard curve.

b. Phloroglucinol. The alcohol eluant of the phloroglucinol band was reacted with Lindtz reagent. After 5 min the optical density was read at 500 mmc. The concentration of phloroglucinol in the eluant could then be computed from a standard curve.

RESULTS

In the first series of experiments, uniformly labeled C^{14} fructose was infiltrated into radish tubers. The activities of the anthocyanin and its degradation products are shown in Tables 1 and 2. Tables 1 and 2 differ in that they are the results of two separate experiments repeated approximately one week apart. The same experiments were repeated using carboxyl labeled acetate as the active substrate. The results of these experiments are shown in Table 3.

TABLE 1

Activity of anthocyanins and degradation products isolated after C^{14} fructose infiltration of radish tubers

	Total activity in counts per second less background
Anthocyanin	30,450 c/sec
Glucose	3,150 c/sec
Cyanidin chloride	10,300 c/sec
Protocatechuic acid	165 c/sec
Phloroglucinol	30 c/sec
	Specific activity in counts/second per mg of carbon
Cyanidin chloride	1754 c/s/mg c
Protocatechuic acid	1312 c/s/mg c
Phloroglucinol	611 c/s/mg c

TABLE 2

Activity of anthocyanins and degradation products isolated after C^{14} fructose infiltration of radish tubers

	Total activity in counts per second less background
Anthocyanin	21,300 c/sec
Glucose	1,800 c/sec
Cyanidin chloride	9,200 c/sec
Protocatechuic acid	43 c/sec
Phloroglucinol	20 c/sec
	Specific activity
Cyanidin chloride	1150 c/s/mg
Protocatechuic acid	780 c/sc/mg
Phloroglucinol	400 /sc/mg

TABLE 3

Activity of anthocyanins and degradation products isolated after $CH_3C^{14}OOH$ in infiltration of radish tubers

	Total activity in counts per second less background
Compound	
Anthocyanin	24,300 c/sec
Glucose	225 c/sec
Cyanidin chloride	4,800 c/sec
Protocatechuic acid	41 c/sec
Phloroglucinol	22 c/sec
	Specific activity in counts/second per mg of carbon
Cyanidin chloride	1003 c/s/mg c
Protocatechuic acid	440 c/s/mg c
Phloroglucinol	1754 c/s/mg c

DISCUSSION

It is evident from the results that the anthocyanin may derive its entire structural skeleton from sugars. These results would confirm the results of Neish, in general, although they differ somewhat in detail. Neish *et al (11, 12)* in studies of the synthesis of quercetin in buckwheat found that the addition of uniformly labeled sucrose, uniformly labeled both the A and B rings of quercetin. The intact buckwheat plants used in their experiments were allowed to photosynthesize during the incorporation of radio-active sucrose. In our case there was no opportunity for photosynthesis.

As the radish tuber was isolated from photosynthetic tissues, respiration could be expected to continue during infiltration. It is likely that the carbohydrate pool was metabolized to give rise to acetate and other intermediates. Thus upon the addition of radio-active sugars the carbohydrate was undergoing glycolysis and the compounds directly derivable from glucose and fructose were made with higher specific activities. The products which were formed from the end products of glycolysis would be more diluted by in situ intermediates giving a phloroglucinol (or ring A) of a lower specific activity. Underhill *et al (11)* found that quercetin was formed more readily from acetate than from sugars.

If the results of the radio-active acetate experiments are compared with those of the fructose it is apparent that the results are consistent. The radio-activity in this case was predominantly incorporated into phloroglucinol whose synthesis has been postulated by the head to tail linkage of acetate. The activity which was found in the glucose can only be explained by the assumption of a dynamic pathway of glycolysis. It is obvious that the reverse reaction must be small in magnitude since the total counts in the glucose attached to the anthocyanidin was extremely small when compared to the results of fructose incorporation.

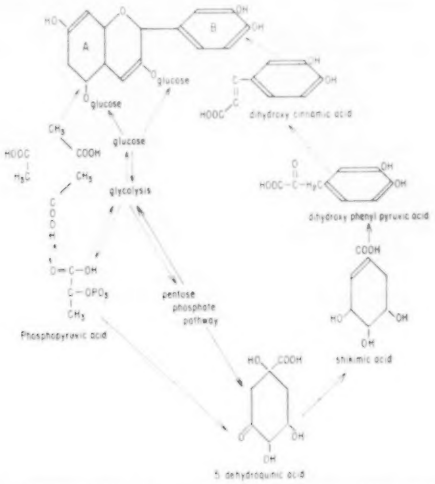

Figure 2. Intermediates in the formation of cyanidin diglucoside.

The results of this experiment would indicate that the C-3, C-6 unit as well as the attached glucose are formed more directly from sugars than they are from acetate. The phloroglucinol unit is formed more directly from acetate than from the carbohydrate pool in the radish tissue. Figure 2 illustrates the proposed origin of the various carbons in the molecule.

SUMMARY

Uniformly labeled fructose and carboxyl labeled acetate were incorporated into isolated radish tubers to observe the biosynthesis of anthocyanin from these compounds. The anthocyanin produced was systematically degraded to glucose and cyanidin, and further to phloroglucinol and protocatechuic acid. All of the compounds were labeled from both fructose and acetate. When fructose was used the two glucose moieties as well as the C-3, C-6 units of the cyanidin were more heavily labeled than ring A. When carboxyl labeled acetate was the active infiltrate, the anthocyanin isolated was generally active although less so than in the sugar labeling experiments. Ring A in these experiments (carboxyl labeled acetate) was relatively more active than in the previous experiments.

The results strongly suggest that in the biosynthesis of flavonoid compounds in non-photosynthetic tissues, ring A is derivable directly from head to tail acetate linkages, and the remainder from sugars through the shikimic acid pathway.

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A STUDY OF A SPECTROPHOTOMETRIC METHOD FOR THE FLAVOR EVALUATION OF INSECTICIDE TREATED VEGETABLES [a]

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Synthetic organic insecticides cause off flavors in vegetables as well as other foods *(1, 2, 4)*. As use of these insecticides has increased 600% in the past 2 decades *(3)*, the need for rapid objective methods of flavor evaluation of insecticide treated foods has become more pronounced. Therefore, this investigation was designed to develop an objective method for evaluating flavor in insecticide treated vegetables.

Work of Sondheimer, Phillips, and Atkins *(7)* suggested that flavor differences could be characterized by absorption spectrophotometry. They found that the absorbency, at 265 mmc, of a 40 ml n-hexane or Skellysolve-B extract of 5 g of bitter carrots was greater than similar extracts of non-bitter carrots. They obtained a degree of correlation between "height of 265 mmc peak" and carrot bitterness as judged by a flavor panel.

EXPERIMENTAL

History of vegetable crops. In 1956, Chantenay carrots and Purple Top White Globe turnips were grown on soils which had been treated with 12 applications of insecticides in both 1952 and 1953 and six applications in 1954. Insecticides used and total pounds applied per acre were as follows: aldrin (40% wp), 30; BHC (12% gi-wp), 30; chlordane (50% wp), 120; DDT (50% wp),30; dieldrin (50% wp), 30; Dilan (50% wp), 120; lindane (25% gi-wp), 30; heptachlor (25% wp), 120; and Toxaphene (40% wp), 150.

In 1957, Carmelcross corn and the same varieties of carrots and turnips were grown in soils which were treated shortly before planting. These soils had no previous insecticide treatments. Insecticides used and pounds applied per acre were as follows: aldrin (50% wp), 4; BHC (12% gi-wp), 4.16; and dieldrin (50% wp), 5.

Processing of samples. Test crops in 1956 were frozen using the following procedure. Samples were washed, hand peeled, and diced into one-half inch cubes. Carrots were blanched in boiling water for 3 min and turnips for 2 min. The blanched vegetables were packed in frozen food cartons, overwrapped with waxed paper, frozen, and stored at 0° F (−17.8° C).

Test crops in 1957 were canned and processed according to recommendations of The National Canners Association *(6)* and stored at temperatures ranging from 65° F (18.3° C) to 95° F (35° C) for subsequent testing.

Flavor panel evaluation. Within 6 months after processing, samples were evaluated using a trained flavor panel of from 10 to 15 judges.

A multiple comparison procedure was used in evaluating test samples, a control or reference standard was used in all evaluations. Included among the test samples was a coded control sample. Two replicate evaluations were made on each sample.

The flavor data were analyzed using an analysis of variance and the least significant difference between panel scores calculated. If no significant flavor difference (95%

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confidence limit) was found between a treated sample and the control, the sample was considered "similar" in flavor to the control. If a significant flavor difference was found between a treated and control sample, the sample was designated as "different" from the control. Spectrophotometric examinations were made only on those samples which were significantly different in flavor from the control. The one exception was aldrin treated turnips grown in 1957.

Lyophilization of samples. Vegetables were lyophilized on a Vir-tis "Freezemobile" in order to obtain sufficient solvent extractable material from small size samples. In comparison with other methods of drying biological materials, lyophilization does the least damage (*5, 8*). Pressure of lyophilization was 20–50 mc and total time was 17 hr in order to insure complete drying. Lyophilized samples were stored at 0° F (−17.8° C) in bottles which had been evacuated and flushed with nitrogen 3 times. Condensates, or liquid frozen around the outer surface of the lyophilizer condenser, were collected and stored at 0° F (−17.8° C) for subsequent testing.

Solvent extraction. Three to 4-g portions of lyophilized control and treated vegetables were weighed to within 0.003 g and extracted for 24 hr in a Soxhlet extractor using the following solvents:

1. 250 ml of peroxide-free ethyl ether, bp 34.6° C.
2. 250 ml of 95% ethyl alcohol, bp 83.1° C.
3. 250 ml of reagent grade carbon disulfide, bp 46.3° C.

One hundred ml of thawed condensates were extracted with 50 ml of carbon disulfide on a shaker at a 45 degree angle for 2 hr.

Spectrophotometric examinations. Solvent extracts of control and treated samples were diluted in order that they would not absorb too strongly in various spectral regions. Spectra of comparable control and treated samples were compared. Carbon disulfide extracts of turnips grown in 1957 were evaporated to dryness and weighed. Solutions of equal concentrations were made and examined.

TABLE 1

Summary list of crops, insecticide treatments, extracting solvents, and spectral regions examined

Crop	Insecticide	Solvents	Spectral regions
Carrots—1956	Lindane, dieldrin, Toxaphene, Toxaphene, dilan	Ethyl ether 95% ethyl alcohol	Visible Ultra-violet
Turnips—1956	Aldrin, dieldrin chlordane, DDT	Ethyl ether 95% ethyl alcohol	Visible Ultra-violet
Carrots—1957	BHC, aldrin	95% ethyl alcohol, carbon disulfide	Infrared Visible Ultra-violet
Corn—1957	Aldrin	Carbon disulfide	Infrared
Turnips—1957	BHC, aldrin, dieldrin	95% ethyl alcohol, carbon disulfide	Infrared Visible

RESULTS AND DISCUSSION

Differences in infrared spectra, as indicated in Figure 1, were observed with carbon disulfied extracts of condensates from samples with flavor differences in 11 out of 15 comparisons. It was felt that these differences may have been due to differences in extract concentrations. Therefore, extract weights were determined after evaporating to dryness. Extracts were made up to equal solution concentration and examined. Four of six comparisons showed differences.

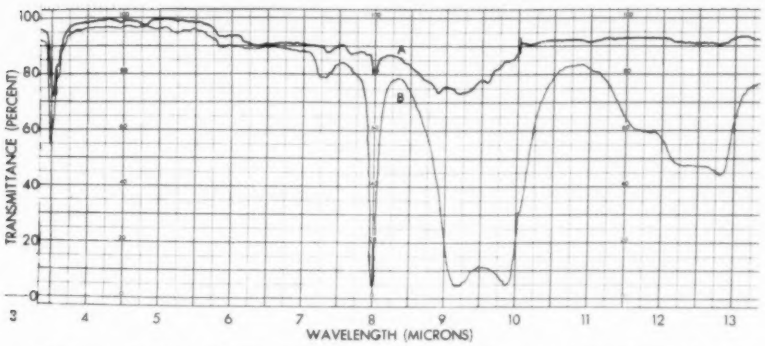

Figure 1. Typical infrared spectra found in 11 out of 15 comparisons of carbon disulfide extracts of condensates from the lyophilization of flavor-different untreated and insecticide treated vegetables. A. Untreated. B. Insecticide treated.

All comparisons of carbon disulfied extracts of condensates from 1957 control and aldrin treated turnips without flavor differences did not show infrared differences. This is significant, for if procedural or chance differences were involved in condensate spectral differences found in this study, at least one of the sets of control and aldrin samples should have shown spectral differences. Therefore, it seems likely that condensate infrared differences can be associated with flavor differences.

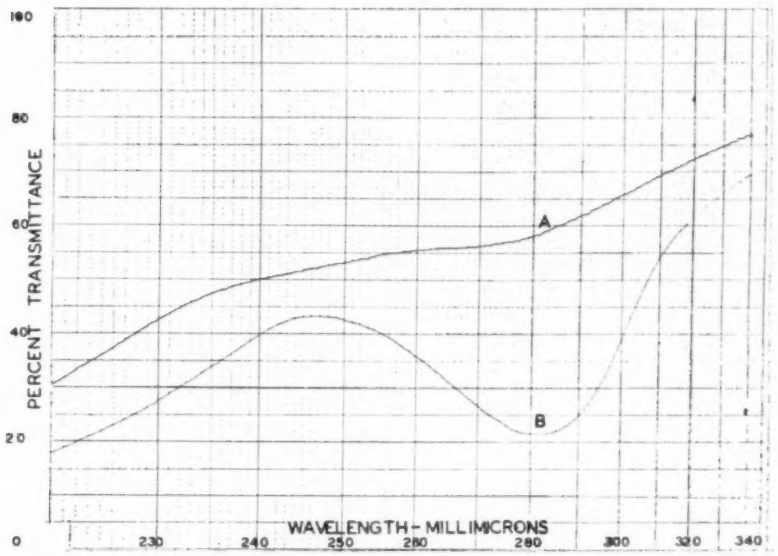

Figure 2. Typical ultra-violet spectra found in 3 out of 7 comparisons of ethyl alcohol extracts of untreated and BHC treated carrots and turnips. A. Untreated. B. Insecticide treated.

With one exception, comparisons between solvent extracts of lyophilized samples did not show any spectral differences. Three out of 7 comparisons between ethyl alcohol extracts of control and BHC treated carrots and turnips had spectral differences as shown in Figure 2. At the present, no explanation can be given for this.

Results suggest that future studies should be concerned with collection and fractionation of vegetable volatiles and their examination by infrared spectrophotometry.

SUMMARY

Untreated and insecticide treated vegetables with flavor differences were lyophilized. Solvent extracts of the lyophilized vegetables and their condensates obtained during lyophilization were examined in the visible, ultraviolet, and infrared regions for spectral differences.

Differences in spectra were observed with carbon disulfide extracts of the condensates in the infrared region. No differences were found in extracts of the lyophilized vegetables in the different spectral region with one exception. Ethyl alcohol extracts of control and BHC carrots and turnips had different spectra in the ultra-violet region.

Acknowledgment

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THE OXIDATION OF TERPENES. I. MECHANISM AND REACTION PRODUCTS OF D-LIMONENE AUTOXIDATION

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The deterioration of essential oils and products containing essential oils has been a serious problem in the food flavor and essential oil fields for many years. A study of terpene autoxidation would give a more thorough understanding of the reactions occurring between the terpene, oxygen, and the catalyst. Although these reactions are known to be quite complex *(2, 4, 5, 6, 7, 13, 14, 15, 16, 18, 19)*, involving initiation of a free radical chain reaction, chain propagation with oxygen participation, and chain termination, a systematic study of several of the more important variables would help clarify some of the more cryptic aspects of the over-all problem. The natural choice for a substrate is limonene since this terpene is one of the most widely distributed and abundant hydrocarbons found in the plant kingdom. It occurs in numerous volatile oils and is present in some as the main constituent, especially in the citrus oils. D-Limonene has been identified in oil of orange (about 90%), lemon, mandarin, lime, grapefruit, bergamot, neroli, petitgrain, elemi, caraway (40%), dill, fennel, celery (60%), erigeron, and orthodon *(8)*.

EXPERIMENTAL

The course of the autoxidation reactions was followed by oxygen uptake which was determined by conventional techniques using a Warburg respirometer *(17)*. The main compartment of the reaction flask contained 0.2 ml (1.23×10^{-3} moles) of D-limonene unless otherwise noted, and the center well of the vessel contained 0.2 ml of distilled water. It was found to be extremely difficult to obtain accurate and meaningful readings of the manometers when the oxidations were carried out in the absence of water. This is probably due to some anomalous effects of vapor pressure encountered between the water in the manometer fluid and the substrate. In later experiments a modified Erlenmeyer flask was employed to provide a greater surface area for oxidative attack, and the water was added directly to the substrate. [Similar techniques using aqueous dispersions have been reported by Borglin *et al (5)*]. The authors were unable to distinguish any difference between oxidations carried out in the two flasks. All autoxidation experiments were conducted in duplicate, and frequently a number of replicate determinations were carried out.

A control experiment was run concurrently with all manometric examinations. The control vessel contained all the constituents of the actual reaction mixtures examined with the exception that an inert gas, nitrogen, filled the vessel instead of air.

The gas-liquid chromatographic apparatus employed to separate the products of the autoxidation of D-limonene was an Aerograph model A-90-C.[b] Columns were constructed of stainless steel tubing, ¼ inch o.d. and 10 feet in length. The support material used throughout this study was Sil-O-Cel C-22 diatromaceous earth firebrick (30-60 mesh). Fractions of this were sieved to size and further graded by sedimentation in water, after which they were dried at 110° C for 17 hr. The liquid phase was introduced by deposition from benzene solution, the packing being heated in a stream of helium to a temperature 20-30° C higher than any used in the subsequent experiments.

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The column was contained in an air thermostat; the carrier gas flow was controlled by a precision reducing valve and measured by a soap bubble flow meter situated at the column outlet. Temperature and flow control were sufficiently good to make possible the use of a four-channel, hot wire katharometer that proved virtually noiseless when connected with a 1 mv recording potentiometer. Sample injection was made by a microsyringe, and the column preheater was maintained at 50° C above the temperature at which the column was operated.

Separations were carried out with the aid of three stationary liquid phases: beeswax (mostly esters of C_{26} and C_{28} acids), LAC-2-R446 *(11)* and LAC-4-R777 *(12)*. The materials were applied to the solid support in the amount of 25% (w/w).

The parameters of operation were as follows: column temperature 150° C; helium flow rate 90 ml/min; sample volume 5 mcl; recorder chart speed 30 in/hr. The column outlet was maintained at atmospheric pressure.

The D-limonene used in this study was a commercial product [c] having the following physical constants: n_D^{25} 1.4700; α_D^{25} + 98.59°. The literature values *(13)* are as follows: n_D^{20} 1.4726; α_D^{25} + 101°. Examination of this material by means of gas-liquid chromatography employing a stationary phase of LAC-2-R446 revealed that the limonene had the following proximate composition (based upon integration of the areas under the appropriate peaks): D-limonene 97.70%; α-pinene 0.37%; β-pinene 0.21%; myrcene 1.43%; and unknown compound 0.27%.

The D-carvone employed was a commercial product [d] having the following physical constants: n_D^{25} 1.4960; α_D^{25} + 56.72°. The literature values *(8)* are as follows: n_D^{20} 1.4995; α_D^{25} + 59.57°.

No physical constants were available for the *trans*-carveol [e] as the amount obtained was far too small to permit their accurate evaluation. Evaluation of this material by gas-liquid chromatography revealed that it was better than 90% pure.

The crystalline hemin [f] was used without further purification.

The ultraviolet absorption spectra of limonene and its oxidation products were obtained at room temperature using a Beckman DK-2 recording spectrophotometer.

RESULTS AND DISCUSSION

The course of the autoxidation of D-limonene in a typical experiment is presented in Figure 1. An initial induction period or lag phase was consistently observed, followed by a period of higher but constant oxidation rate. The duration of the induction period was dependent upon the amount of limonene present and decreased at higher levels of limonene (Figure 1). In general, the induction periods ranged from 24 to 48 hr under the conditions obtaining in these experiments. The reactions were followed manometrically for 8 days or up to 5.1 mol % of oxygen uptake. With increased time and greater oxygen uptake, the measurements became less accurate due to the marked increase in viscosity of the reaction mixture. After some 10–15 days, the limonene became yellow and began to resinify thus making it unsuitable for further manometric studies. Widmark and Blohm *(19)* in a comparative study of the autoxidation of Δ^3-carene, α-pinene, β-pinene and (+)-limonene allowed the reactions to proceed for some 30 days and observed a similar increase in viscosity of the limonene during this time.

The maximum autoxidation rate of D-limonene plotted as a function of the amount of limonene present in the reaction vessel is presented in Figure 2. From these data it may be observed that the maximum autoxidation rate is

[c] Matheson, Coleman and Bell Lot No. 5405141.

[d] *Ibid.*, Lot No. 392092.

[e] The *trans*-carveol was obtained from Dr. William L. Stanley, U.S.D.A., Pasadena, California.

[f] Armour and Company.

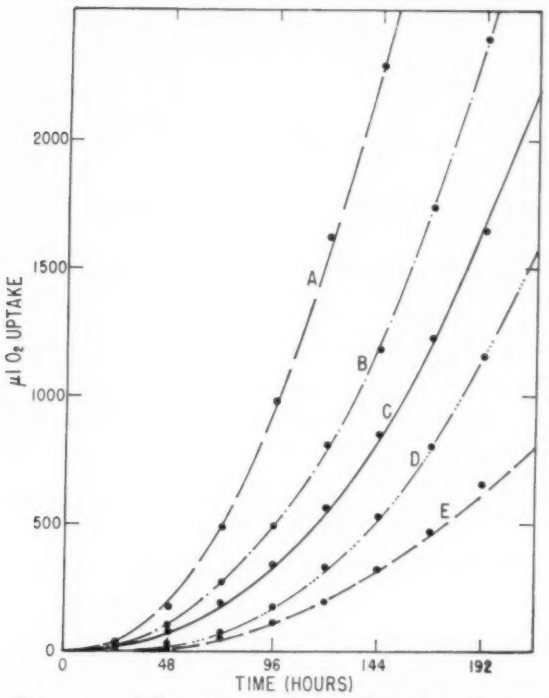


Figure 1. The course of limonene autoxidation. The effect of limonene level on the rate of oxygen uptake at 30° C. Limonene level: (A) 6.17 x 10^{-3} moles; (B) 3.09 x 10^{-3} moles; (C) 1.85 x 10^{-3} moles; (D) 1.23 x 10^{-3} moles; (E) 6.17 x 10^{-4} moles.

linear up to levels of 1.85 x 10^{-3} moles of limonene. Beyond this point the rate falls off sharply indicating that some other factor has become rate limiting. In the region between 6.17 x 10^{-4} and 1.85 x 10^{-3} moles of limonene, the rate of oxygen absorption does not appear to be limited by oxygen diffusion into the liquid phase, since the rates increase with increasing amount of substrate.

Figure 3 presents the results of a series of experiments designed to show the effects of various autoxidation promoters upon the induction period and rate of autoxidation of D-limonene. In this series of experiments 0.1 ml (6.17 x 10^{-4} moles) of D-limonene was used. The addition of 10 mg of hemin to the reaction mixture virtually eliminated the induction period of the reaction *(16)*. There was a slight induction period observed on addition of 10 mg of benzoyl peroxide to the reaction medium. The rates of the two reactions are virtually identical after this initial lag phase has passed *i.e.*, 18.5 mcl O_2 uptake/hr. When oxygen was admitted to the reaction vessel and air was excluded, a 24-hr induction period was observed. A duplicate vessel filled with air also showed a similar 24-hr induction period. After this time, the rates were the same, within the limits of experimental error, as indicated by the parallel curves (Figure 3). Addition of 10 mg of copper

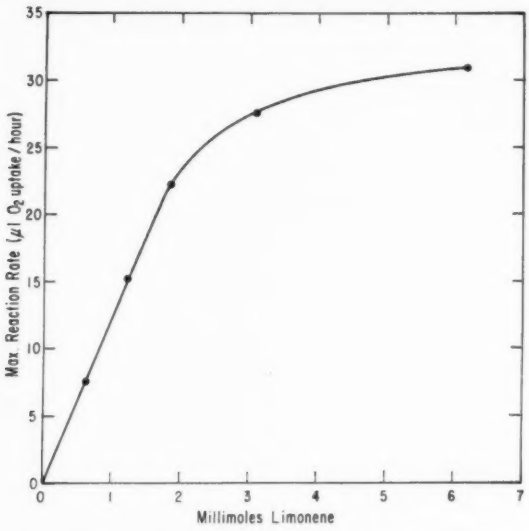


Figure 2. Maximum autoxidation rate for limonene as a function of limonene level at 30° C.

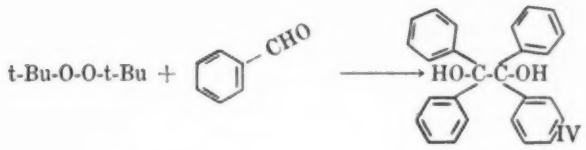
sulfate to the reaction mixture increased the induction period some additional 12 hr. After this time, the rate of autoxidation was identical to those exhibited by the vessels containing no catalyst, but only air or pure oxygen (Figure 3). The rate of limonene autoxidation in these three vessels was 13.9 mcl 0_2 uptake/hr; some 24.9% less than the rate in the vessels containing the free radical promoters. A possible explanation for the increased length of the induction period on addition of cupric salts may lie in the fact that copper stabilizes the alkoxy radical formed in the chain initiation reaction. Being paramagnetic, copper could conceivably combine with a free radical to form a stable complex. The following scheme may possibly occur:

$$\underset{\text{I}}{Cu^{++}} + \underset{\text{II}}{R\bullet} \rightleftharpoons \underset{\text{III}}{Cu^{++}\bullet R} \qquad (A)$$

If III is formed initially this would remove a reactive free radical from the chain initiating mechanism. Assuming that the equilibrium for reaction (A) lies far to the left, I and II are then formed and the olefin autoxidation reaction proceeds accordingly. There is some precedent for this postulated mechanism in the work of Kharasch and Fono *(10)*. These researchers reported that when t-butyl peroxide decomposes, it normally produces acetone and methyl radicals, *viz.*:

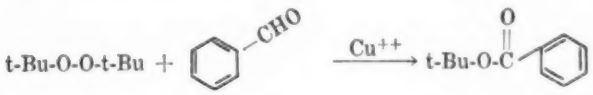
$$\text{t-Bu-O-O-t-Bu} \longrightarrow [\text{t-Bu-O}\bullet] \longrightarrow CH_3CO\text{-}CH_3 + CH_3\bullet \qquad (B)$$

There has been no evidence to indicate more than transitory existence of the t-butoxy radical (indicated above in brackets). When t-butyl peroxide is reacted with benzaldehyde, IV is formed:



(C)

However, in the presence of cupric ion, the above reactants yield t-butyl benzoate:



(D)

The obvious conclusion is that the t-butoxy radical, t-Bu-O•, must have been formed and immediately stabilized by the Cu^{++} present in the reaction medium.

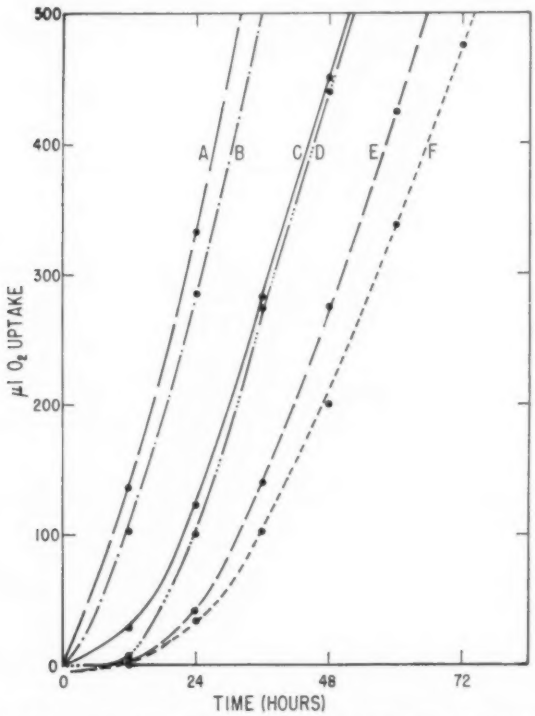


Figure 3. Effects of various autoxidation promoters upon the induction period and rate of limonene autoxidation at 30° C. (A) hemin; (B) benzoyl peroxide; (C) air; (D) oxygen; (E) copper sulfate; (F) ultraviolet light.

On exposure of limonene to 10 seconds of moderately intense ultraviolet light of 2540 Å and subsequent autoxidation, it was noted that there was a lengthening of the induction period followed by a significant decrease in the rate of oxygen uptake (rate: 11.6 mcl O_2 uptake/hr). It may well be that ultraviolet radiation alters the course of chain initiation, and propagation. Such phenomena are known, and one example, the autoxidation of α-terpinene to give polymeric peroxides has been discussed by Russell *(14)*.

A control flask filled with nitrogen run concurrently with all the above experiments, as expected, showed no gas uptake or evolution.

The autoxidation reaction was next examined at 4 different temperatures and an energy of activation calculated from these data. On the basis of data taken at 30; 35; 40; and 45° C, the energy of activation was calculated to be 8.9 Kcal/mol. A plot of the data in the form of the Arrhenius equation is presented in Figure 4. Walling *(18)* indicates that, in general, energies

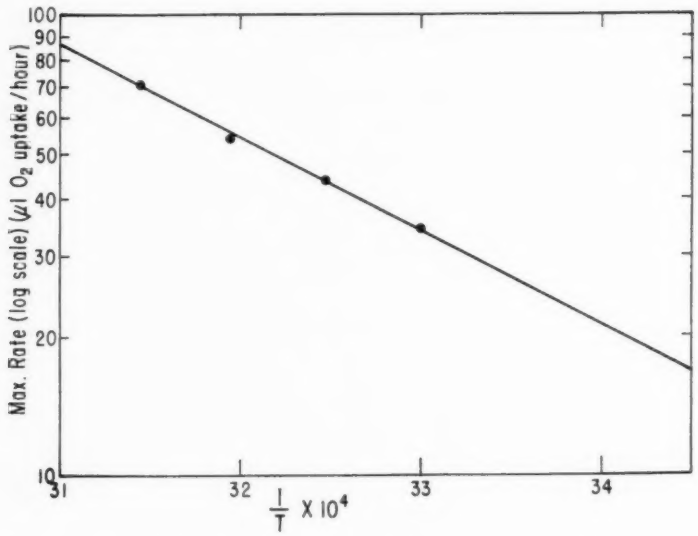


Figure 4. Plot of the Arrhenius equation (maximum reaction velocity (log scale) vs reciprocal of absolute temperature).

of activation for autoxidation reactions of olefins are in the range of from 5 to 8 Kcal; thus, the above value appears reasonable. This is the minimum activation energy for this reaction. In going from 30 to 45° C a decrease in oxygen solubility in the liquid phase occurs. The reaction rate at the higher temperatures is probably slower than it would be if the oxygen solubility did not decrease with increased temperature.

It was hoped to be able to follow the rate of the reaction by means of ultraviolet spectrophotometry, but it was soon demonstrated that constant removal of samples from the reaction flask caused aberrations in the kinetic

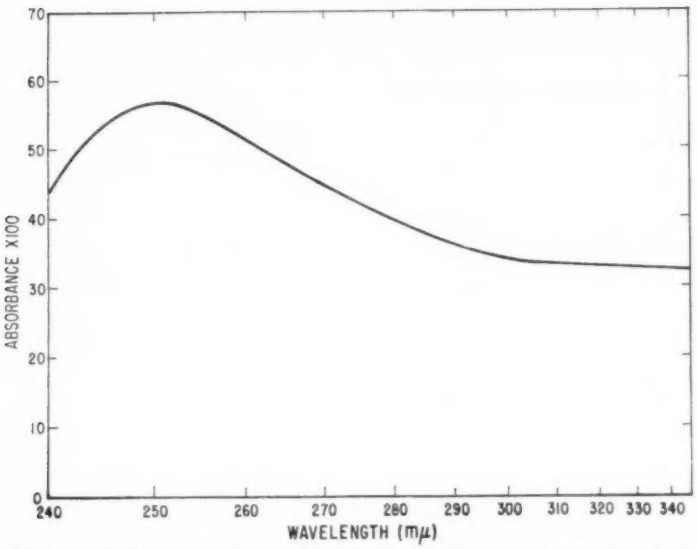


Figure 5. Difference spectrum of products of limonene autoxidation. Limonene concentration: 1 mg/100 ml n-heptane.

data. A clear difference spectrum was obtained from the reaction products which showed a broad absorption maximum near 250 mmc (Figure 5). The precise nature of the material or materials producing this peak has not as yet been elucidated.

Daily examination by means of the technique of gas-liquid chromatography of a reaction mixture similar to those studied in the manometric vessels showed the progressive formation of 7 major oxidation products and a number of minor ones.

Analysis of these autoxidation products presents considerable difficulties, for examinations must be made before the reaction medium has polymerized to any appreciable extent. The reaction must be examined at or near the early stages of oxidation where conversion to products is limited. It was noted above that total oxygen uptake was of the order of 5 mol %; only extremely small amounts of conversion can take place during the period when examination is permissible. Undoubtedly, a number of products polymeric in character were not recorded on the chromatogram.

When the products of the autoxidation of limonene, after 15 days of reaction, were examined by means of gas-liquid chromatography employing a stationary phase of beeswax, 4 peaks were evident on the chromatogram. Gas chromatograms with either LAC-2-R446 or LAC-4-R777 as the stationary liquid phase showed 14 major peaks (Figure 6).

Assignment of various peak identities was made by determination of the corrected retention volumes, V_R^0, *(1)* of known compounds and comparison

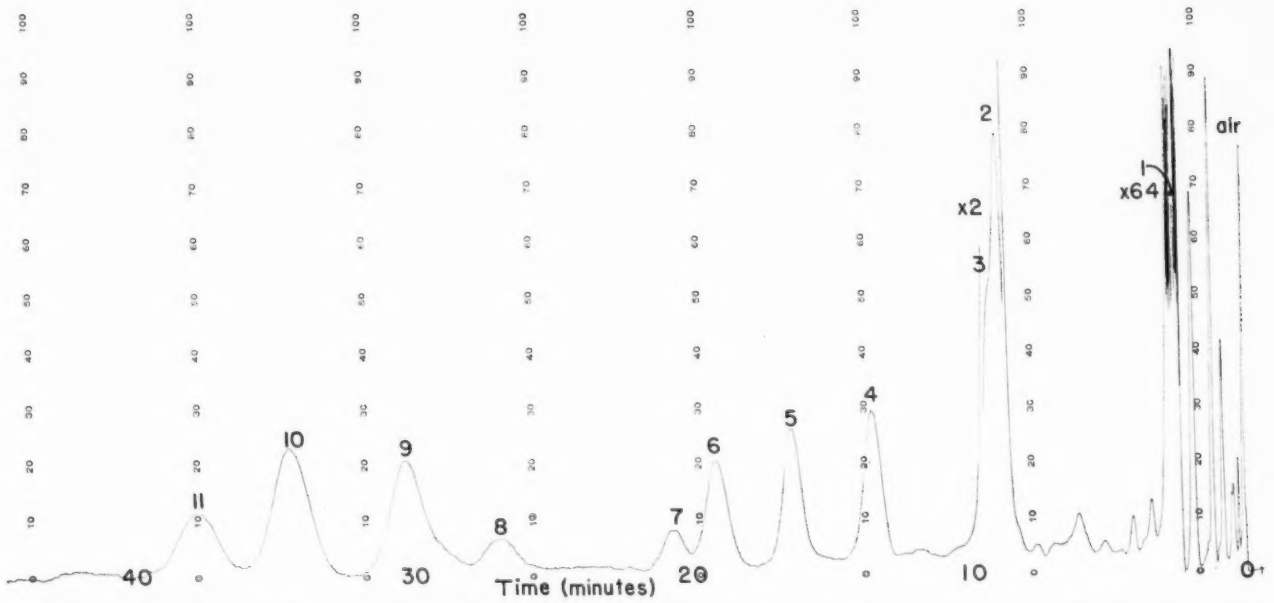


Figure 6. Chromatogram of the autoxidation products of D-limonene. Sample size: 5 mcl; temperature: 150° C; helium flow rate: 90 ml/min; stationary phase: LAC—4—R777 on a support of Sil-O-Cel C-22 (30-60 mesh), 25% (w/w); stainless steel column 10 feet by ¼ inch; 1 mv recording potentiometer; chart speed: 30 inches/hour. (See Table 3.)

TABLE 1

Relative retention volumes of the components present in autoxidized limonene.[1] Stationary phase: beeswax [2] (Limonene = 1.00)

	V_R^O/V_R^O		
Peak	Unknown	Known	Compound
1	1.00	1.00	D-limonene
2	1.90	1.90	*trans*-carveol
3	2.37	.....	.....
4	2.90	2.99	D-carvone

[1] After 15 days of autoxidation.
[2] Temperature 150° C; helium flow rate 90 ml/min.

TABLE 2

Relative retention volumes of the components present in autoxidized limonene.[1] Stationary phase: LAC–2–R446 [2] (Limonene = 1.00)

	V_R^O/V_R^O		
Peak	Unknown	Known	Compound
1	1.00	1.00	D-limonene
2	1.34	.....	.....
3	1.55	.....	.....
4	2.04	.....	.....
5	2.40	.....	.....
6	2.92	.....	.....
7	3.38	3.33	linalool
8	6.71	.....	.....
9	10.7	10.4	D-carvone
10	12.5	12.2	*trans*-carveol
11	13.6	.....	.....

[1] After 15 days of autoxidation.
[2] Temperature 150° C; helium flow rate 90 ml/min.

TABLE 3

Relative retention volumes of the components present in autoxidized limonene.[1] Stationary phase: LAC–4–R777 [2] (Limonene = 1.00)

	V_R^O/V_R^O		
Peak	Unknown	Known	Compound
1	1.00	1.00	D-limonene
2	3.60	3.64	citronellal
3	3.73	(3.91)	(linalool)
4	5.47	.....	.....
5	6.66	.....	.....
6	7.79	.....	.....
7	8.43	8.37	citronellol
8	10.9_8	10.9_7	citral
9	12.3_8	12.5_2	D-carvone
10	14.0_8	13.6_4	*trans*-carveol
11	15.4_2	.....	.....

[1] After 15 days of autoxidation.
[2] Temperature 150° C; helium flow rate 90 ml/min.

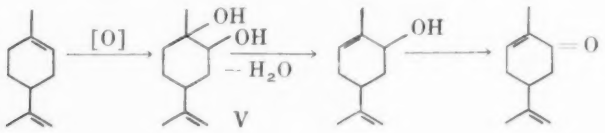
with those for the unknown peaks. In this manner, a tentative identification of some of the components present was achieved. These results were confirmed by an enrichment procedure in which known compounds were added, one at a time, to fresh portions of the oxidized reaction mixture and re-examined by gas-liquid chromatography. Data are presented in the form of relative retention volumes (V_R^o/V_R^o) *(1)* (Tables 1-3). It should be noted that it is experimentally impossible to distinguish between two compounds whose relative retention volumes differ by 7% or less since they will appear on the chromatogram as a single, united peak; differences of 10 to 15% in relative retention volumes will show peaks that are united, e.g., shoulders, or doublets; and differences of 20% or more are necessary for complete separation of peaks *(3, 9)*.

In order to be well within the bounds of experimental error, an arbitrary limit of agreement not to exceed 3% was established. Compounds with values for corrected relative retention volumes not agreeing to within 3% of each other are enclosed in parentheses.

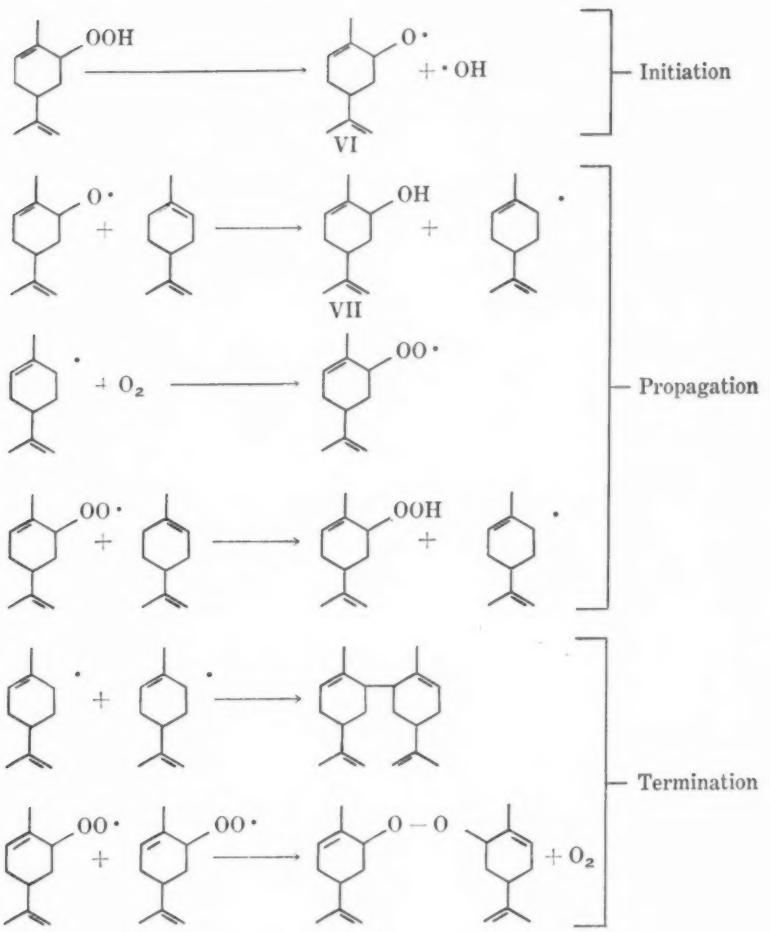
As a further check on identity, relative retention volumes were evaluated on three different liquid phases, beeswax (Table 1), LAC-2-R446 (Table 2), and LAC-4-R777 (Table 3). By employing 3 stationary liquid phases and examining the reaction products on each, 3 sets of corrected retention volumes were obtained. Using this information (Tables 1–3) a tentative identification of some of the autoxidation products of D-limonene was made. These data indicate that D-carvone, *trans*-carveol, and a number of unidentified compounds are among the major autoxidation products. After several days an odor suggestive of D-carvone was detected in the autoxidized mixture.

Relative retention volumes for some 70 known terpene compounds were determined in order to identify the products of the reaction mixture, and it was found that there was no satisfactory correlation, except as noted in Tables 1-3, between any of these values and those obtained from the chromatograms of the autoxidation products.

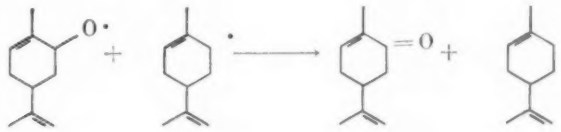
The observation that carvone and carveol are present as autoxidation products is in accord with the findings of Blumann and Zeitschel *(4)* who have shown that the oxidation of limonene in moist air proceeds in what Simonsen *(15)* calls "a somewhat unexpected manner" to give carvone and carveol. It was presumed that the primary product of the reaction was the glycol (V), from which the alcohol, carveol, was formed by loss of water:



In the light of present knowledge, the above mechanism does not appear to be adequate or even correct. It seems more likely that the autoxidation occurs via a free radical mechanism. Assuming that a free radical mechanism is involved, it is possible to postulate a free radical chain reaction that will account for the formation of carveol and carvone. In general terms this reaction can be pictured as follows:

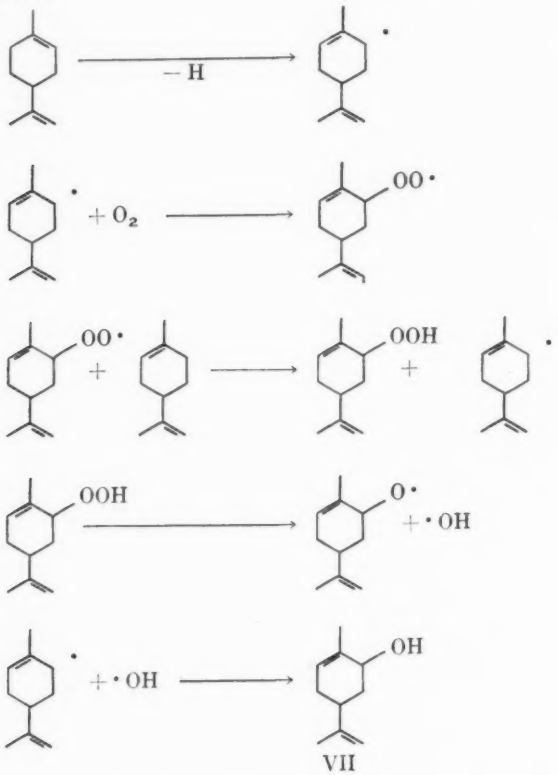


The alkoxy radical (VI) can undergo reactions which do not lead to chain propagation. One such reaction could account for the formation of the ketone, carvone (VIII):



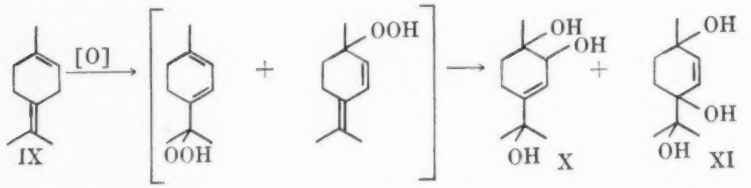
VIII

Flores and Morse *(7)* postulate a chain mechanism that differs slightly from the above:



VII

Formation of polyols from terpene autoxidation is well known. Borglin, *et al (5)* describe the case of terpinolene (IX) which does not give an identifiable hydroperoxide but rather a mixture of crystalline triols; (X) and (XI):



Formation of the alcohols and polyols could account for the lack of agreement between retention volume data for the unknown peaks and the values for the known terpene compounds examined. Additionally, although autoxidation initially may give hydroperoxide in high yields *(6, 18)*, the

hydroperoxides can subsequently attack the double bond of another molecule by both polar and radical processes. As a result, prolonged oxidation generally yields a complex mixture of products containing several olefin residues per molecule in addition to hydroperoxides. The possibility of peroxide addition to the double bond, rather than hydrogen abstraction, is a potential complication and leads to alternate copolymerization chains *(18)*. The intermediate allylic radical, being a resonance hybrid of two structures, may add oxygen at either end of its electronic system and lead to rearranged products *(18)*. Finally, cis-trans isomerization occurs as well, with the hydroperoxides having the more stable trans-configuration *(18)*.

At present only 3 compounds have been tentatively identified as being products of the autoxidation of limonene: 2-limonyl hydroperoxide *(6)*, carvone, and carveol *(4)*. The mechanisms presented above are consistent with current theory and account for the formation of these products.

SUMMARY

Studies of the autoxidation of D-limonene have revealed that the reaction appears to proceed via a free radical mechanism. The course of the autoxidation displays the typical induction phase followed by a period of constant oxidation rate. The maximum autoxidation rate is directly proportional to limonene levels up to 1.85×10^{-3} moles (under conditions of the experiments described herein).

Addition of free radical promoters such as hematin and benzoyl peroxide virtually eliminate the induction period or lag phase of the autoxidation reaction; while addition of copper sulfate and exposure to moderate ultraviolet light increase the lag phase.

The energy of activation calculated from the experimental data for these reactions is 8.9 Kcal/mol.

Examinations of the products of this reaction by means of gas-liquid chromatography indicate that there are some eleven products of the autoxidation, and two of the products have been tentatively identified as being D-carvone and *trans*-carveol. A mechanism is presented which accounts for the formation of these compounds.

Ultraviolet difference spectra for the total reaction mixture during autoxidation show a broad absorption maximum near 250 mmc.

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STATIONARY LIQUID PHASES FOR USE IN GAS-LIQUID CHROMATOGRAPHY SUITABLE FOR THE SEPARATION OF THE COMPONENTS OF ESSENTIAL OILS[a]

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A number of attempts have been made to find a suitable stationary phase for use in gas-liquid chromatography (GLC) that would permit the satisfactory separation of the bulk of the constituents of essential oils *(2)*. Previous workers in the field have separated groups or classes of compounds, *e.g.*, the citrals *(9)* with success *(1, 3, 5, 6)*. However, to this author's knowledge, few have succeeded in employing GLC to examine an intact oil and to achieve adequate resolution of the major components present. In this connection the work reported by Howard *(4)* in 1956 on the constituents of hop oil is interesting. Howard implied that an increase in resolution can be expected over that obtained in his preliminary work and did, in fact, get indications of some 18 components in the oil. No mention was made of the stationary phase employed in this study *(4)*. Bernhard *(2)* demonstrated the presence of only five distinct peaks in whole lemon oil using GLC employing a liquid phase consisting of a polyethylene glycol, *Carbowax 600*. It now appears probable that the detectors used in this early study, single filament type, were insufficiently sensitive to pick up the signals from compounds present in minute amounts. With the improved equipment used in this study, four filament type detectors, larger numbers of peaks could have been detected.

The most powerful experimental parameter available to the researcher working with gas-liquid chromatographic equipment employing katharometer type detectors is the proper choice of the stationary liquid phase. This study is concerned with the quest for such phases and the results obtained.

EXPERIMENTAL

The apparatus employed to separate the constituents of the essential oils was an Aerograph model A-90-C[b] equipped with a hot wire 4-channel katharometer assembly. Columns were constructed of stainless steel, ¼ in o.d. and 5 ft in length unless otherwise noted. The support material used throughout this study was Sil-O-Cel C-22 diatomaceous earth firebrick[c] 30 to 60 mesh. Fractions of this material were sieved to size and further graded by sedimentation in water, after which they were dried intensively for 18 hr. The various liquid phases were introduced by deposition from solutions in appropriate solvents, *viz.*, ethanol, acetone, ether, benzene, etc. The materials were applied to the support in the amount of 25% by weight. The packing was then heated for 18 hr in a stream of helium to a temperature higher than any used in the subsequent experiments.

[a] Supported in part by a grant from the Sunkist Growers, Inc., Los Angeles, California.

[b] Manufactured by Wilkins Instrument and Research, Inc., Walnut Creek, California.

[c] Product of Johns-Manville Company.

When the commercial detergent [4] was employed as the stationary phase, no solid support was used. The detergent was heated in an oven at 150° C for 24 hr, sieved to 30-60 mesh and packed into the stainless steel tube. This preheating removes a considerable amount of volatile materials from the detergent which otherwise would have to pass through the detector.

The column was contained in a thermostated air bath. The power supply to the air bath was further regulated by passage through a 250 VA *Sola* constant voltage transformer. The carrier gas flow was controlled by a precision reducing valve and measured by a soap bubble flow meter situated at the column outlet. Temperature and flow control were sufficiently good to make possible the use of a 4-channel, hot wire katharometer that proved to be virtually noiseless when connected with a 1 mv recording potentiometer. Sample injection was made by a microsyringe, and the sample preheater was maintained at 50° C above the operating temperature of the column. The column outlet was kept at atmospheric pressure.

RESULTS AND DISCUSSION

The principal goal of this study was to find those compounds which when employed as a stationary liquid phase would show the largest number of components or peaks for an essential oil, *viz.*, lemon oil, on a gas-liquid chromatogram. Naturally, this is a rather arbitrary index of performance, but it proved to be the most direct and simple method at hand to solve the problem. The separation of minor components can be accomplished after the major separation has been achieved.

Since the appearance of the first publication in this series *(2)*, the author has scanned some 40 liquids as possible stationary phases for use in GLC to examine essential oils and to separate and identify their constituents. A list of those compounds tested is presented in Table 1 together with the source of these materials.

When cold-pressed lemon oil was examined by GLC using glycerol as the stationary liquid phase, 20 distinct peaks were observed; methyl abietate gave 15 peaks; di-nonyl phthalate showed 14 peaks; Dow 710 (a silicone oil containing phenyl groups) showed 10 peaks; and *Carbowax 1500* showed 15 peaks. The newer polyesters such as Craig polyester adipate gave 27 peaks, and Craig polyester succinate showed 22 components. During the course of these investigations serendipity played an important role. The adipate polyester of diethylene glycol partially cross-linked with pentaerythritol (sold commercially as LAC-2-R446) *(7)* was tried as a stationary phase. Although Lipsky and Landowne *(7)* had employed this liquid successfully for the separation of esters of unsaturated fatty acids, no one has reported on its use to separate the components of essential oils. When cold-pressed lemon oil was examined by GLC employing LAC-2-R446 as the stationary liquid phase, 32 distinct peaks were evident. Examination of orange oil under similar conditions revealed some 19 distinct peaks, lime oil showed 17 peaks, and grapefruit oil showed 16 peaks. A hexane extract of the common mint plant revealed 22 distinct peaks. It may be noted that LAC-2-R446 is superior in this application to its sister compounds LAC-1-R296 (the adipate polyester of diethylene glycol) and LAC-4-R777 (the succinate polyester of diethylene glycol) *(8)*. Use of LAC-1-R296 gave 22 peaks for lemon oil, and LAC-4-R777 gave 29 peaks. Apparently cross-linking with pentaterythritol increases the discriminatory effect of LAC-2-

[4] Tide.

TABLE 1

Stationary liquid phases examined in this study

Compounds	Source	Relative Separation Efficiency
AMINES		
Triethanolamine	Eastman Kodak Co., Rochester, N.Y.	−
ESTERS		
Di-*n*-butyl phthalate	Eastman Kodak Co., Rochester, N.Y.	+
Di-*n*-octyl phthalate	Eastman Kodak Co., Rochester, N.Y.	+
Di-nonyl phthalate	K&K Laboratories, Inc., Long Island City, N.Y.	++
Di-isodecyl phthalate	Ohio Apex Co., Los Angeles, Calif.	+
Di-decyl phthalate	Ohio Apex Co., Los Angeles, Calif.	+
Di-decyl adipate	Ohio Apex Co., Los Angeles, Calif.	+
Di-*n*-octyl sebacate	Eastman Kodak Co., Rochester, N.Y.	+
Methyl abietate	Eastman Kodak Co., Rochester, N.Y.	++
Tri-(2, 6, 8-trimethyl-4-nonyl) borate	U. S. Borax and Chemical Corp., Los Angeles, Calif.	−
Sucrose acetate isobutyrate (SAIB)	Eastman Chemical Products, Inc., Kingsport, Tenn.	+
Tri-cresyl phosphate	Ohio Apex Co., Los Angeles, Calif.	+
Tween 20 (polyoxyethylene sorbitan monopalmitate)	Atlas Powder Co., Wilmington, Del.	+
Tide	Procter & Gamble Co., Cincinnati, Ohio	−
Craig polyester adipate	Wilkens Instrument and Research, Inc., Walnut Creek, Calif.	+++
Craig polyester succinate	Wilkens Instrument and Research, Inc., Walnut Creek, Calif.	+++
LAC-1-R296	Wilkens Instrument and Research, Inc., Walnut Creek, Calif.	++
LAC-2-R446	Wilkens Instrument and Research, Inc., Walnut Creek, Calif.	++++
LAC-4-R777	Wilkens Instrument and Research, Inc., Walnut Creek, Calif.	++++

TABLE 1 (continued)

Beeswax (chiefly esters of C_{26} and C_{28} acids)		+
GLYCOLS		
Glycerol		+++
Silver nitrate in glycerol (saturated solution)		−
Silver nitrate in ethylene glycol (saturated solution)		−
Ethylene glycol		−
Polypropylene glycol	Wilkens Instrument and Research, Inc., Walnut Creek, Calif.	+
Carbowax 400	Union Carbide Chemicals Co., New York, N.Y.	++
Carbowax 600	Union Carbide Chemicals Co., New York, N.Y.	++
Carbowax 1500	Union Carbide Chemicals Co., New York, N.Y.	++
HYDROCARBONS		
Mineral oil		+
Squalene	Eastman Kodak Co., Rochester, N.Y.	−
Apiezon L	James G. Biddle Co., Philadelphia, Penna.	+
Apiezon M	James G. Biddle Co., Philadelphia, Penna.	+
Apiezon N	James G. Biddle Co., Philadelphia, Penna.	+
SILICONES		
SF-96 (40 centistokes)	General Electric Co., Schenectady, N.Y.	+
SF-96 (100 centistokes)	General Electric Co., Schenectady, N.Y.	+
DC-550	Dow Corning Co., Midland, Mich.	+
Dow 710	Wilkens Instrument and Research, Inc., Walnut Creek, Calif.	+
DC high vacuum grease	Dow Corning Co., Midland, Mich.	+
MISCELLANEOUS		
Flexol Plasticizer 8N8	Beckman Instruments Co., Fullerton, Calif.	+

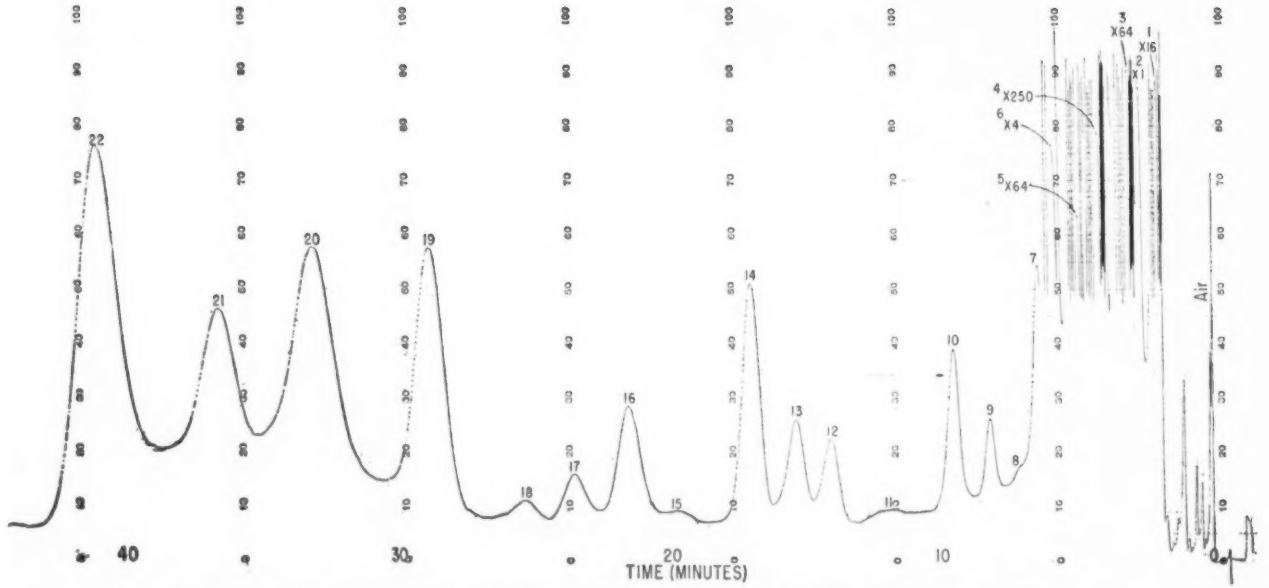

Figure 1. Chromatogram of the components of a California cold-pressed lemon oil. Sample size: 20 mcl; temperature: 150° C; helium flow rate: 60 ml/min; stationary phase: LAC-2-R446 on a support of Sil-O-Cel C-22 (30-60 mesh), 25% by weight; stainless steel column 10 feet in length by ¼ inch o.d.; 1 mv recording potentiometer; chart speed: 30 inches/hour.

R446 toward subtle differences in structure, *i.e.*, double bond positions, endocyclic rings, etc.

Use of LAC-2-R446 poses some rather engaging possibilities to the essential oil researcher. A typical chromatogram of the components present in whole California cold-pressed lemon oil is presented in Figure 1 to demonstrate the extent of separation. The powerful effects exerted by the proper selection of the right liquid phase for a given separation is very graphically illustrated by reference to Figure 2. This shows a chromatogram of lemon oil separated on a column of the commercial detergent.[4] It can be noted that

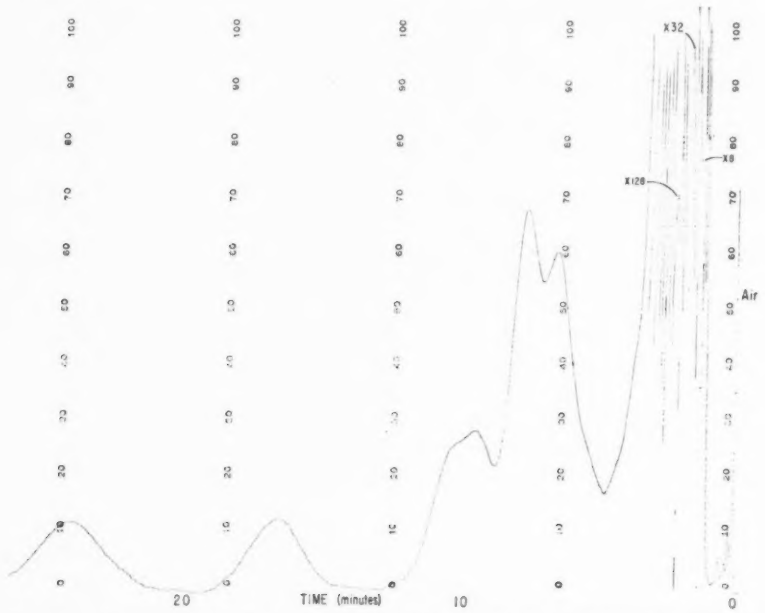

Figure 2. Chromatogram of the components of a California cold-pressed lemon oil. Sample size: 20 mcl; temperature 150° C; helium flow rate: 90 ml/min; stationary phase: TIDE, 30-60 mesh; stainless steel column 5 feet in length by ¼ inch o.d.; 1 mv recording potentiometer; chart speed: 30 inches/hour.

only 8 ill-defined peaks are evident. The experimental conditions obtaining for this chromatographic run were similar to those employed using the LAC-2-R446 column and illustrated in Figure 1. Further work on the components in lemon and orange oil indicated that LAC-2-R446 can be used to separate the common terpenes quite successfully, and at relatively low temperatures, *e.g.*, 80-100° C. A plot of log relative retention volume *vs.* boiling point of the terpene was not linear for all the terpenes examined. This indicated a selective discrimination by this liquid phase over those previously examined for minor structural differences in these hydrocarbons, *i.e.*, the position of a double bond, and it also indicated that vapor pressure effects alone do not govern the degree of separation achieved.

SUMMARY

Forty liquids were examined as stationary liquid phases to be used in gas-liquid chromatography for the separation of essential oils. The compounds that gave the best separation of the components of cold-pressed lemon oil were LAC-2-R446, LAC-4-R777, and Craig polyester adipate. Typical chromatograms are presented for the best and poorest cases of separation, and a table of all compounds investigated is included.

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OXIDATION OF TISSUE LIPIDS IN COOKED PORK[a]

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Oxidative rancidity has been shown to be responsible for off odors and flavors which develop during storage in muscle tissues subjected to thermal treatment *(10)*. These organoleptic changes can be correlated with rancidity when assays are performed on the whole tissue *(15)* but not when fat alone, extracted with the usual solvents, is used *(13, 14)*. It appears, therefore, that bound lipids are contributing markedly to the reactions responsible for rancid odors and flavors in cooked meats.

Protein bound phospholipids are known to be important food constituents involved in the deteriorative reactions which take place during processing and storing *(7)*. These substances are among the more labile components of foods. Their role in the deterioration of a few commodities such as eggs and milk has been studied *(7)*. Little is known, however, about their contributions to spoilage mechanisms in muscle tissues.

Published work is still deficient on analyses of the individual phospholipids of organs and tissues of various species of animals. Dawson *(2)* has reviewed studies on the phospholipid composition of rat tissues. Lecithin and phosphatidylethanolamine predominated in skeletal muscle while phosphatidylserine and phosphoglycerocetals occurred in smaller amounts. Christl *(1)* demonstrated the presence of phosphoglyceracetals (formerly called plasmalogens) in bovine skeletal muscle.

Very highly unsaturated fatty acids are quite widely distributed in small quantities as constituents of the fatty acid moiety of phospholipids *(6)*. It is often found that the phospholipids from a particular tissue are appreciably more unsaturated than triglyceride fats from the same source *(7)*. Their presence renders the lipid more susceptible to oxidation. Such oxidation is, undoubtedly, a major factor in deteriorative reactions leading to strongly flavored degradative products at an early stage.

Protein bound phospholipids cannot be extracted readily with the usual fat solvents. More polar solvents such as methanol, ethanol or butanol are required and even then some of the tenaciously bound lipids are not liberated *(7)*. In view of this, the importance of selecting a suitable procedure for measuring oxidative rancidity becomes apparent.

Peroxide determinations have been used to measure rancidity in various fats and oils as well as in more complex food commodities in which the fat is intermingled with other constituents as protein or carbohydrate. Muscle tissue is an example of the latter case. When peroxide tests are performed

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on this tissue, extraction of the fat is a necessary preliminary step. In earlier studies on cooked pork *(13, 14)* oxidative changes were not observed when peroxide determinations were used as a method of assay. Palatability ratings decreased but rancidity was not thought to be an important factor in organoleptic changes. A test which could be performed directly on the tissue itself might offer a means of measuring oxidative changes in protein bound lipids which are not extractable with the ordinary fat solvents.

The 2-thiobarbituric acid (TBA) test offers such a means for measuring rancidity. It can be performed directly on the meat tissue without the necessity of extracting the fat and is a sensitive test for the decomposition products of highly unsaturated fatty acids *(5)*. It would be expected to measure oxidative changes which cannot be detected by peroxide determinations made on extracted fat. Current procedures for carrying out the TBA test, although useful in studying the development of oxidative rancidity, are of empirical nature, being expressed in arbitrary units varying from laboratory to laboratory. Sinnhuber and Yu *(8)* and Tarladgis *et al (9)* recently proposed quantitative procedures using a reference standard.

The present research was designed to compare rancidity tests made on whole tissue versus extracted fat and to determine the lipid fraction primarily responsible for the oxidation.

PROCEDURE

Preparation of meat. Fresh pork hams were gound, packed into 307 x 113 C-enameled cans (175 g per can) and cooked in a steam bath to an internal temperature of 70° C *(10)*. After cooking, the cans were opened immediately, the meat ground again, thoroughly mixed and stored at refrigeration temperature (5-7° C) for subsequent assays.

Chemical methods for testing lipid oxidation. Two methods were used to measure oxidative rancidity: (a) the Wheeler peroxide method as modified by Watts *et al (12, 13)* and (b) the 2-thiobarbituric acid test as outlined by Turner *et al (11)* with a slight modification in that optical densities were read on the Evelyn photoelectric colorimeter using a 540 filter. Peroxide numbers represent milliequivalents of peroxide per 1000 g of fat. Data for Turner's method are reported as "TBA value" which is the optical density of the colored complex formed by reacting the TBA reagent with the 5.0 g sample of meat. Some TBA tests were performed on fat extracted from the meat either with carbon tetrachloride or a chloroform-methanol solution *(3,4)*. These organic solvents were exaporated under nitrogen before performing the TBA test.

Extraction of total lipids and separation of neutral fat and proteolipids. The method of Folch *et al (3,4)* was used for extracting total lipids from a meat sample and separating the neutral fat and phospho- and proteolipids. Briefly the method involves homogenization of the meat tissue with a chloroform-methanol solution, filtration to obtain a clear extract, and separation of the neutral fat phase from the phospho- and proteolipid phase by allowing a measured volume of extract to stand overnight over a layer of water. The latter phase separates as "interfacial fluff".

RESULTS AND DISCUSSION

Experiments to compare TBA values and peroxide numbers. Earlier studies *(13, 14)* on oxidative changes in frozen cooked pork have shown no correlation between palatability ratings and peroxide numbers. Since the TBA test can be performed directly on the meat tissue, it may measure oxidative changes in tissue lipids which do not appear in peroxide determinations made on the extracted fat.

Data obtained from these two rancidity tests, determined on the same sample of meat, both raw and cooked, are given in Figure 1. The ordinates

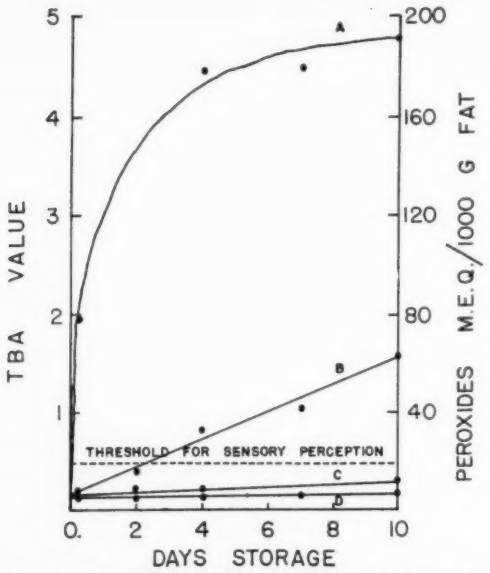

Figure 1. Comparison of TBA values versus peroxide numbers of refrigerated raw and cooked pork. Legend: A. TBA value, cooked meat. B. Peroxide number, extracted fat from cooked meat. C. Peroxide number, extracted fat from raw meat. D. TBA value, raw meat.

were so chosen that threshold sensory values for the two tests coincide on the graph. Cooked samples were assayed as soon as possible after cooking. The time interval between the termination of the heating period and the analysis was approximately three hours. Even with this short storage period the TBA value of cooked sample was beyond the 0.4 to 0.6 level which has been shown to be the range at which rancidity becomes detectable organoleptically *(15)*. The TBA increased rapidly to a maximum in approximately 4 days. On the other hand, not until after the second day of storage did peroxide numbers pass the value of 20 which is considered to be the point at which rancid odors and flavors are perceptible in pork fat *(13)*. Raw samples showed no appreciable change in TBA values with storage and only slight variations in peroxidase numbers. As previously pointed out *(15)*, ferric hemochromogens formed during cooking are believed to catalyze the oxidation of tissue lipids.

Further comparisons of peroxide numbers and TBA values were made on samples of meat which had been held in freezer storage for approximately 15 months. These data are reported in Table 1. TBA values for all cooked freezer-stored samples were beyond the point of acceptability while peroxide numbers were still low.

Comparative data on the two tests in cooked meats are available only for pork at this time. However, it has been established that TBA values increase equally rapidly following the cooking of beef, veal, lamb and chicken *(10)*. On the other hand, the triglyceride composition of the meat might be expected

TABLE 1

Peroxide numbers and TBA values of freezer stored pork

Internal cooking temperature	Rancidity after 15 months of freezer storage	
	TBA values	Peroxide numbers
Raw	0.34	4.1
60° C	2.0	7.6
70° C	2.6	7.3
85° C	2.4	4.4

to influence the rate of peroxidation, pork and chicken being very different from beef or lamb in this respect. These discrepancies between peroxide numbers and TBA values support the hypothesis that the latter are not primarily a measure of the oxidation of triglycerides extractable with the usual fat solvents.

In order to explore this possibility peroxide determinations on extracted fat were compared with TBA tests on both extracted fat and meat tissue. The triglycerides were extracted with carbon tetrachloride from a 25-g sample of meat and the amount of fat present was determined by drying an aliquot. An amount of the carbon tetrachloride-fat solution corresponding to that in 5-g tissue was used for the TBA test and a peroxide determination was made on a suitable aliquot of the remaining solution. Meat tissue used for the TBA test was from the same lot as that used for the fat extraction.

The results are shown in Table 2. Peroxide values increased only slightly during the 7-day storage period and were always below the threshold for sensory detection of rancidity. TBA values on the extracted fat from 5 g of tissue, like the peroxide values, remained low. On the other hand, TBA values determined on 5 g of the whole tissue showed a many fold increase after cooking.

TBA values of lipid fractions. A preliminary experiment was performed to compare the TBA value of triglycerides with that of total lipids from the same sample of cooked, refrigerated pork. The triglycerides were extracted with carbon tetrachloride and the total lipids with chloroform methanol. TBA values were 3.3 for the total lipids as compared to 0.9 for the triglycerides.

In a second, more complete experiment, total lipids were extracted from two samples of cooked pork, one a rancid control, the other having been treated with 0.5% sodium tripolyphosphate antioxidant. Both samples were

TABLE 2

Comparisons of rancidity tests made on extracted fat versus whole tissue

Samples and storage intervals	TBA values of tissue	TBA values of extracted fat	Peroxide numbers
Raw: 2 days	0.21	0.10	6.3
5 days	0.23	0.23	9.0
Cooked: 2 days	2.3	0.14	10.2
5 days	2.2	0.21	10.4
7 days	2.5	0.35	12.9

taken from the same mixed lot of raw pork and were cooked three days prior to the fat extraction. The phospho- and proteolipids were separated from the neutral fat by the method of Folch *et al (3, 4)*. TBA tests were performed on both phases. See Table 3. Data given represent TBA values on extracted lipid in 1 g original tissue. Since 5 g of meat are used in Turner's method, these figures would have to be corrected to approximate that procedure.

Data show that the oxidative reaction in the rancid sample is much more intense in the phospho- and proteolipid phase although these lipids represent only a small proportion of the total weight of lipid. The TBA reaction per g of lipid is approximately 30 times as great for the phospho- and proteolipids as for the neutral fats. On the other hand, in the antioxidant treated sample, TBA values were lower in the nonglycerides than in the neutral fat. The water soluble antioxidant used was presumably capable of making intimate contact with tissue lipids.

TABLE 3

Rancidity in pork lipid fractions

Lipid fraction	Weight of lipid/g tissue		TBA values/g tissue	
	Rancid sample	Antioxidant-treated sample	Rancid sample	Antioxidant-treated sample
	g	*g*		
Total lipids	.272	.289	0.65	0.13
Neutral fat	.246	.263	0.20	0.14
Phospho- and proteolipids	.026	.026	0.59	.06

Results of this and preceding experiments necessitate some modifications in our concepts of the extent and importance of "rancidity" in meats. If it is conceded that oxidation of phospho- and proteolipid fractions should also be classed as rancidity, then the flavor deterioration usually associated with the storage of cooked uncured meats comes under this heading. We have used the term "tissue rancidity" to differentiate this from the more familiar neutral fat oxidation. The nonglyceride fraction not only oxidizes more rapidly in cooked meats, but also produces off odors which are qualitatively different from those associated with neutral fat oxidation. This might be expected in view of the highly unsaturated nature of the lipids involved. Our knowledge of tissue rancidity is as yet very fragmentary, but with the improvement in methods for extraction and separation of tissue lipids, it should be possible to explore this subject much more adequately in the future.

SUMMARY

Comparisons of rancidity tests on extracted fat versus whole tissue showed that the lipid fraction primarily involved in the rapid oxidative reaction which takes place in cooked meats can be extracted with a mixture of chloroform and methanol but not with neutral fat solvents. TBA tests performed on total lipids fractionated into triglycerides and proteolipids demonstrated that the latter are responsible for the intensive reaction induced by heating muscle tissue.

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UNSATURATED TRIGLYCERIDE AND FATTY ACID LIPOXIDASE ACTIVITIES OF NAVY BEANS, SMALL RED BEANS, PEANUTS, GREEN PEAS AND LIMA BEANS[a]

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Siddiqi and Tappel *(6)* have reported the presence of lipoxidase activity in a number of legumes using linoleate salts as substrates. Earlier these same workers investigating pea lipoxidase *(5)* suggested an implication of lipoxidase action to the production of off odors in underblanched frozen peas. Wagenknecht and Lee *(9)* observed a similar relationship and postulated that the pea lipase produced the substrate for lipoxidase action. However, Koch *et al (3)* found relatively high peroxide values and corresponding "off odors" were produced when navy bean lipoxidase acted on cottonseed oil without a noticeable increase in free fatty acid.

Koch *et al (2)* recently reported that soybeans are capable of oxidizing by apparent lipoxidase action both linoleic acid and the tri-glyceride trilinolein. Presumptive evidence was presented for the existence of two separate enzymes, one which can act on natural triglycerides and which may be responsible for "off odor" development in natural products containing little free fatty acids.

This paper reports further studies on the existence of the two types of lipoxidase activity in other legumes. Also presented are studies on the prevention of off odor development in enzyme inactivated products.

MATERIALS AND METHODS

Enzyme extraction. Dried raw navy beans, peas, small red beans, lima beans, and peanuts ground in a Wiley mill to pass through a No. 20 sieve, and soy flour were used as enzyme sources. The soy flour and ground peanuts were each defatted with several changes of Skellysolve F and solvent was evaporated before use. The ground legumes were extracted with distilled water (5 g per 50 ml) by stirring to keep particles in suspension for 10 min and then insolubles were removed by centrifugation. Calcium chloride (64 mg per ml of extract) was added to the supernatant to precipitate inactive protein. The precipitate was removed by centrifugation and the supernatant further clarified by filtration through Whatman No. 4 filter paper. The filtrates were stored at 4° C until used. Except where specifically stated, extracts were made fresh daily.

Substrates. Substrates used were highly purified trilinolein and linoleic acid obtained from The Hormel Institute, molecularly distilled cottonseed oil (to remove tocopherols), and wheat flour oil (extracted from patent flour with Skellysolve F). Triglyceride substrates were prepared by dissolving sufficient oil in a 50:50 mixture of acetone and 95% ethanol to give 20 mg oil per ml of solution. Presence of the ethanol prevented autoxidation of the oils. Linoleic acid was dissolved in 95% ethanol at a concentration of 10 mg per ml of solution.

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[a] This paper reports research conducted by the Quartermaster Food and Container Institute for the Armed Forces and has been assigned No. 1003 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense.

Reaction mixture. The reaction mixture consisted of 100 ml distilled water, 5.0 ml buffer solution (0.2M), 1 ml substrate solution, and appropriate amount of enzyme extract to give suitable hydroperoxide formation in the desired time interval. Reaction temperature was 20° C.

Lipoxidase assay. The method described by Koch *et al* was followed for measurement of lipoxidase activity. This included hydroperoxide determination by the thiocyanate method of Sumner *(7)*.

RESULTS AND DISCUSSION

Navy beans, peanuts, small red beans, green peas and lima beans were investigated following the same procedures as those reported for soybeans *(2)*. All of these products showed differences in lipoxidase activity characteristics similar to soybeans with some variations as noted below.

pH studies. The effect of pH on lipoxidase activity in navy beans was studied using 4 different substrates, Figure 1.

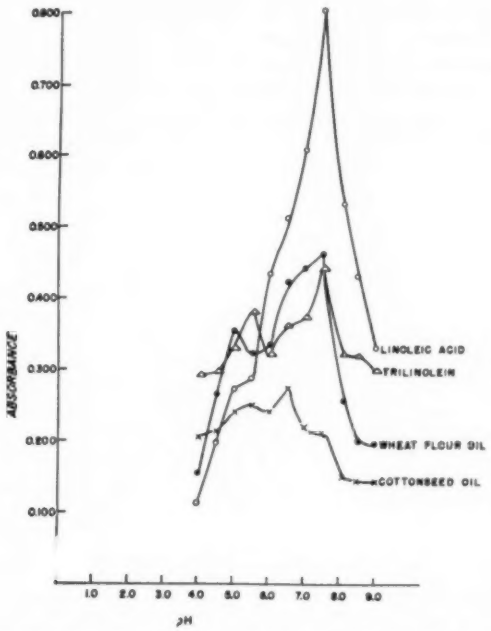

Figure 1. Effect of pH on activity of navy bean lipoxidase for 4 minute reaction time. Reaction mixture: 3.5×10^{-4} M linoleic acid or 2.2×10^{-4} M trilinolein, cottonseed oil, and wheat flour oil, 0.5 ml extract (100 mg/ml), 20° C.

When linoleic acid was used as the substrate, only one sharp peak was observed. Optimum activity occurred at pH 7.5, slightly lower than the pH 8.3 optimum for soybeans; this activity of navy beans decreased more rapidly than that of soybeans above the pH optimum. pH activity curves were also obtained on 3 different triglyceride substrates. The triglyceride active material showed two apparent pH optima for each of the triglycerides.

Using trilinolein, peaks occurred at pH 7.5 and 5.5 with the greater activity at the higher pH optimum, which is the opposite of that observed for soybeans *(2)*. The rate of lipoxidase action was greater on wheat flour oil than on trilinolein at the higher pH optimum despite the fact that flour oil has only about 50% linoleic acid. The lower pH optimum with flour oil was 5.0. Cottonseed oil, which also contains approximately 50% linoleic acid, was the poorest substrate and showed only one definite peak at pH 6.5. These results on the different triglycerides indicate that the triglyceride active material possesses a substrate specificity.

The effect of pH on the lipoxidase activity of peanuts is shown in Figure 2 for three different substrates. With linoleic acid as substrate, a sharp peak occurred at pH 8.1 (similar to soybeans) and a slight hump at pH 6.0. In this product rates of enzyme action on the fatty acid and triglyceride substrates were not so widely separated as in navy beans and soybeans. Two pH optima were observed for each of the two triglycerides tested. Optimum activity occurred at pH 7.5 and 5.5 for the trilinolein substrate with a slightly greater rate of action at the lower pH which is similar to soybeans, but the opposite of navy beans. Cottonseed oil was a good substrate for peanut lipoxidase. Its rate of activity at pH 7.5 was greater on cottonseed oil than on trilinolein. A second peak in the pH curve with cottonseed oil was noted at pH 6.5. This difference in rates of activity on the two triglyceride substrates again indicates a substrate specificity by the legume lipoxidases.

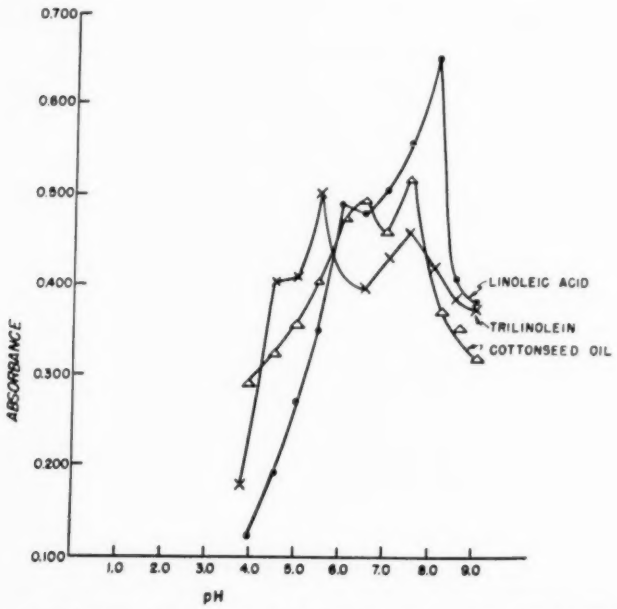

Figure 2. Effect of pH on activity of defatted peanut lipoxidase for 4 minute reaction time. Reaction mixture: 3.5 x 10^{-4} M linoleic acid or 2.2 x 10^{-4} M trilinolein and cottonseed oil, 0.5 ml extract (100 mg/ml), 20° C.

Cottonseed oil was a good substrate for peanut triglyceride active enzyme: it was a poor substrate for the navy bean triglyceride active enzyme.

Figure 2 shows that defatted peanuts (raw Spanish Red variety) contained a substantial amount of lipoxidase activity. Comparing the quantity of extract to give the same absorbance values in a 4-min reaction time, peanuts had approximately 40% of the trilinolein activity of soybeans at pH 5.5. However, peanuts had only 10% of the linoleic acid activity of soybeans at pH 8.1. These values are considerably higher than that reported by Siddiqi and Tappel *(6)* who used pH 7.0 for their assay and found that peanuts on a linoleate substrate had only 1% of the activity of soybeans. Although peanuts are rather poor in linoleic acid activity, next to soybeans they possess the most active trilinolein lipoxidase activity of the 5 legumes reported, Figure 6.

Green pea lipoxidase activity was tested on only two substrates (Figure 3). This product had less total activity than the previous two legumes, but again showed the same two peaks in the pH activity curve with trilinolein as the substrate, i.e., 5.5 and 7.5. Maximum activity on linoleic acid occurred at pH 7.5, the same pH as in navy beans. This is in close agreement with Tappel *et al (8)* who used 0_2 absorption to measure lipoxidase activity on a linoleate substrate. They reported the optimum to be pH 6.9. They did not test for triglyceride activity.

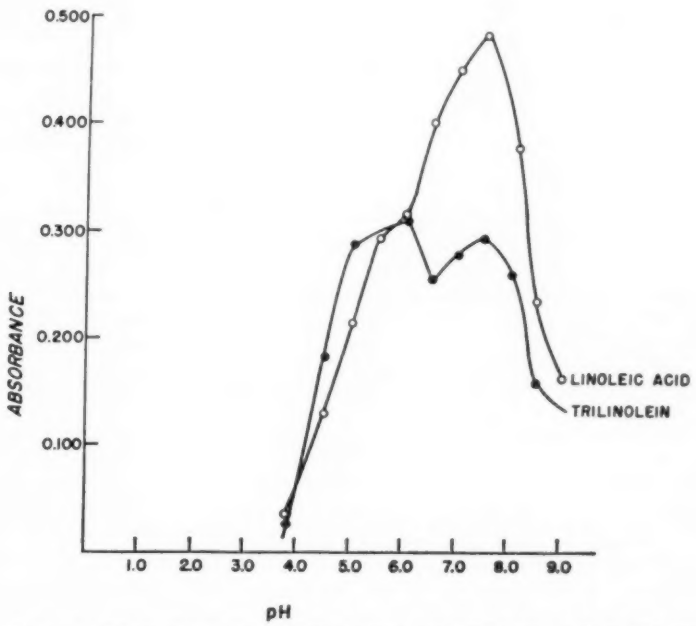

Figure 3. Effect of pH on activity of green pea lipoxidase for 4 minute reaction time. Reaction mixture: 3.5×10^{-4} M linoleic acid or 2.2×10^{-4} trilinolein, 0.5 ml extract (100 mg/ml), 20° C.

Small red beans had a wide difference in the two types of activity, possessing a much greater lipoxidase activity on linoleic acid, Figure 4. Hydroperoxide production from linoleic acid was greatest at pH 7.0, while using trilinolein optima occurred at pH 5.5 and 7.5.

Baby lima beans and large lima beans were the last of the legumes tested. They contained about equal amounts of lipoxidase activity and contained the lowest level of activity yet observed. The pH activity curves for baby lima beans using linoleic acid and trilinolein as substrates are shown in Figure 5. Activity on the trilinolein substrates was very low and showed a broad range of maximum activity between pH 5.5 and 7.0. However, the trilinolein active material was quite labile in the extract and showed a rapid loss in activity which made it difficult to obtain reproducible results.

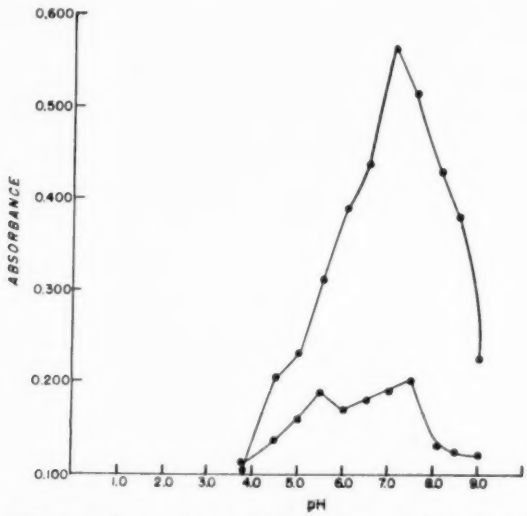

Figure 4. Effect of pH on activity of small red bean lipoxidase for 4 minute reaction time. Reaction mixture: 3.5 x 10^{-4} M linoleic acid or 2.2 x 10^{-4} M trilinolein, 0.5 ml extract (100 mg/ml), 20° C.

It may be possible that this product also has the double triglyceride peak observed in the other legumes.

The enzyme active on linoleic acid in lima beans was more stable and had a sharp optimum at pH 7.5. But it was necessary to use 1 ml of extract, compared to 0.5 ml of extract for the other 4 legumes reported, in order to obtain sufficient hydroperoxide production in a 4-minute reaction time.

Holding the ground lima beans overnight at −4° C did not prevent loss of trilinolein activity (Table 1). It can also be seen, Table 1, that there was a long lag period when 0.5 ml extract was added to a reaction mixture with linoleic acid. When 0.2 ml extract was added, no activity was noted in 16 min. The lag period was no longer apparent when 1 ml of extract was used.

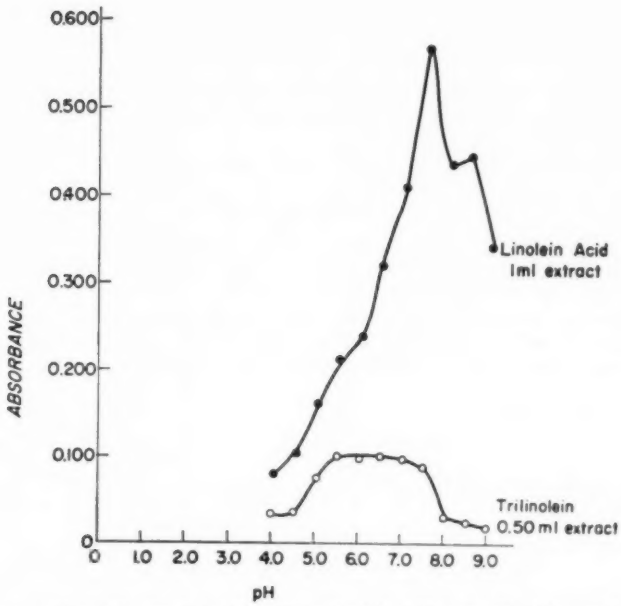

Figure 5. pH activity for baby lima beans, linoleic acid and trilinolein as substrates.

Time studies. Time studies were also carried out on the other 4 legumes. Results are presented in Figures 6 and 7. Using a trilinolein substrate the time course of hydroperoxide production was similar for each legume. However, there was an apparent difference in enzyme content as shown by amount of activity at any given time, Figure 6. Except for peanuts the time curves obtained with a linoleic acid substrate were also similar for the different legumes, but were different from those on trilinolein, Figure 7. A lag period occurred like that reported for soybeans *(2)*. Peanut linoleic acid lipoxidase activity did not show a lag period and followed a time course of reaction similar to the trilinolein active enzyme.

Note that the apparent content of lipoxidase enzyme for trilinolein and linoleic acid shows a considerable variation in the 4 legumes shown in

TABLE 1

Time course of lipoxidase activity of ground lima beans stored overnight at 4° C[1]

Absorbance

Time	Substrate Trilinolein (pH 5.5)	Linoleic Acid (pH 7.5)	
(Min)	*(1.0 ml Ext)*[2]	*(0.50 ml Ext)*[2]	*(1.0 ml Ext)*[2]
2	0.007	0.000	0.152
4	0.004	0.032	0.332
8	0.010	0.164	0.712
16	0.022	0.463	0.760

[1] Reaction mixture: 2.2×10^{-4} M trilinolein or 3.5×10^{-4} M linoleic acid, 0.01 M buffer, 20° C.
[2] Extract concentration: 100 mg/ml.

Figures 6 and 7. Navy beans, which had the highest linoleic acid activity, were third in amount of trilinolein activity. Amount of peanut and green pea lipoxidase activity also shifts positions on the two substrates. The above, together with substrate pH optima differences, would seem to favor the possibility of two different types lipoxidase enzyme and supports similar evidence presented for soybeans *(2)*.

It was also noted that one of the double peaks obtained with the trilinolein substrate occurred at the same pH as the optimum pH for linoleic acid activity for all of the 5 legumes under investigation. This was also observed for soybeans *(2)*. However, for soybeans it was shown that the linoleate active enzyme has little or no activity on the triglyceride substrate. This is also believed to be true for the other legumes. Time studies on the 2 substrates at low enzyme levels of baby lima beans showed distinct differences, Figure 8. A definite induction period occurred for the linoleic acid activity; no lag was observed for equally low trilinolein activity.

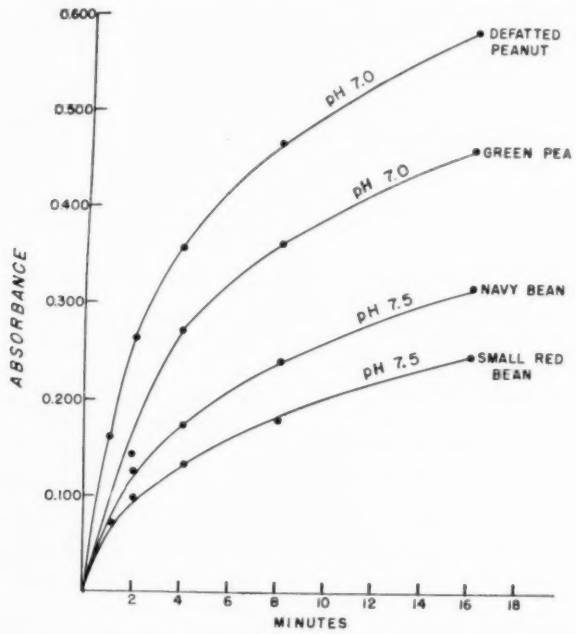

Figure 6. Time curve for 4 legume extracts. Reaction mixture: 2.2×10^{-4} M trilinolein, 0.2 ml extract (100 mg/ml) 20° C.

The reason for the induction period for linoleate active lipoxidase observed with several of the legumes is unknown. Haining and Axelrod *(1)* have reported on the induction period for soybean lipoxidase on a linoleate substrate. They found that minute amounts of hydroperoxide could eliminate the lag in the enzyme oxidation and concluded that hydroperoxide acts as an enzyme activator. An equally plausible explanation of their findings

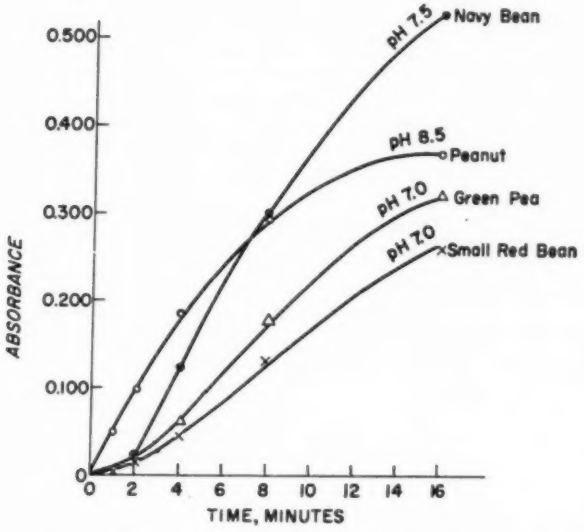

Figure 7. Time studies, linoleic acid substrate, for four legumes.

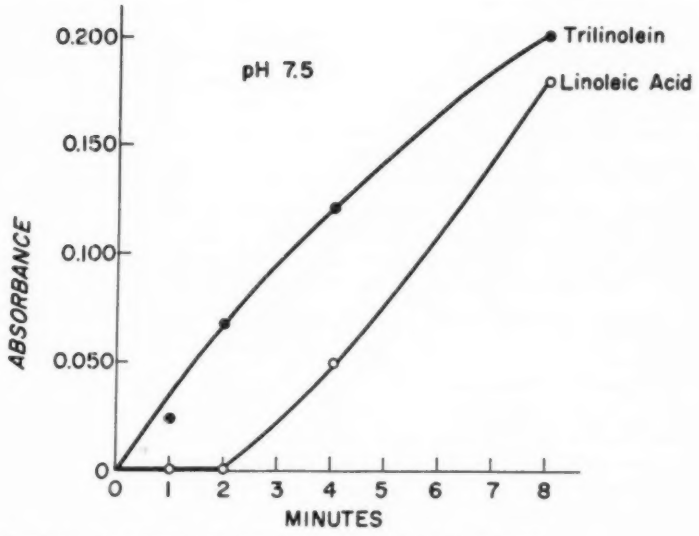

Figure 8. Time studies at low enzyme levels, baby lima beans.

TABLE 2

The effect of hydroperoxides on the induction period in linoleate lipoxidase reactions

A. Baby Lima Beans

	Absorbance		
Initial	0.000	0.051	0.143
(min)	*Increase over initial*		
2	0.000	0.000	0.002
4	0.000	0.000	0.060
8	0.012	0.020	0.107
16	0.097	0.099	0.284

Reaction mixture: 3.5×10^{-4} M linoleic acid, 0.20 ml extract (100 mg/ml), 0.01 M buffer pH 7.5, 20° C.

B. Soybeans

	Absorbance		
Initial	0.000	0.310	0.510
(min)	*Increase over initial*		
1	0.000	0.000	0.015
2	0.000	0.000	0.085
4	0.009	0.052	0.065
8	0.110	0.172	0.235
16	0.319	0.322	0.405

Reaction mixture: 3.5×10^{-4} M linoleic acid, 0.20 ml extract (50 mg/ml), 0.01 M buffer pH 8.3, 20° C.

might be that the hydroperoxide inactivates natural inhibitors present in the enzyme extract. Privett *et al (4)* have shown that the linoleic acid lipoxidase of soybeans is specific for a cis-cis 1,4-pentadiene system, and that conjugated diene systems inhibit the enzyme. It is difficult therefore to see how the linoleate hydroperoxide, which has a conjugated diene structure, can also be an enzyme activator.

In an effort to check on the effect of hydroperoxide on the lipoxidase induction period of baby lima bean and of soybean extracts, time studies were conducted at several initial peroxide levels, Table 2. It appears that a higher peroxide content is required to cause a noticeable decrease in the induction period for soybeans than for lima beans. Although these data do not resolve unequivocally the question of enzyme activation or inhibitor inactivation by hydroperoxide, the very much higher requirement of the soybean extract for equivalent reduction of the induction period, makes the inhibitor inactivation hypothesis more attractive. Soybeans, which contain more lipoxidase activity than lima beans, may also contain more inhibitors of this activity.

Comparison of the hydroperoxide required to shorten the induction period reported here with that reported by Haining *(1)* may be of interest. Standardization of the ferric thiocyanate colorimetric method for determination of peroxide showed than an absorbance of 0.100 is equivalent to 4.5 mc moles of linoleate hydroperoxide in the enzyme reaction mixture. Thus from Table 2, significant reduction of the induction period required 6 mc moles for lima beans and 14 mc moles for soybeans. Haining and Axelrod *(1)* reported a decrease of one-half in the induction period with 1×10^{-3} mc mole of linoleate peroxide and its abolition by 5×10^{-3} mc mole.

One further observation may be made from Table 2. The soybean system whose initial absorbance was 0.510 contained 40 ml of an oxidation mixture containing 35 mc mole of linoleic acid. The peroxide content of this oxidation mixture was 57.5 mc mole, indicating that nearly two-thirds of the original linoleate had been oxidized to a di-peroxide. In other experiments, using small substrate concentrations and oxidizing for long periods, more than 95% of the original linoleate apparently had been carried to diperoxide.

Enzyme stability in extract solutions. As reported above, Table 1, the triglyceride activity in lima beans was quite labile after the whole beans were ground. The other legumes also showed a gradual loss of lipoxidase activity with time after grinding. However, the stability of the enzymes in the extract solutions were the opposite from lima beans. Navy beans, small red beans, and green peas were not as readily inactivated and the linoleic acid activity showed the greatest decrease with time. Peanuts showed the most rapid loss of linoleic acid activity with time, while its triglycerde activity was quite stable when held in solution at 4° C. Thus there seems to be a variation between different legumes in the stability of the two types of lipoxidase activity which adds further evidence for the possible existence of two distinct types of lipoxidase.

SUMMARY

Lipoxidase activities of crude extracts of five different legumes were studied using triglyceride and linoleic acid as substrates.

Variations in pH optima were noted for the different substrates. The triglyceride activity showed two distinct peaks in the pH curves and an apparent substrate specificity for different triglycerides.

Using low level of enzyme activity a lag or induction period was observed with linoleic acid as the substrate for all but one legume tested, while no lag was observed with the trilinolein substrate.

The lipoxidase activity for the two types of substrates also showed variations in stability in the legume extracts.

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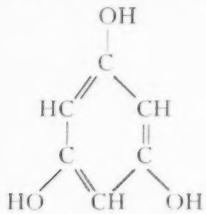
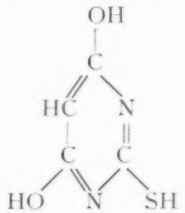
RESPONSE OF EPIHYDRIN ALDEHYDE AND GLYCERALDEHYDE IN THE THIOBARBITURIC ACID TEST FOR FAT OXIDATION[a]

The Kreis and thiobarbituric acid (TBA) tests for oxidative deterioration in fats have a number of characteristics in common. Both tests are carried out in an acid medium and both yield a red pigment when positive. The absorption maxima are 543 and 532 mmc respectively. The reagent of the Kreis test, phloroglucinol, has considerable structural similarity to the enol form of 2-thiobarbituric acid, the reagent of the TBA test.



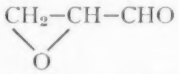
Phloroglucinol



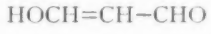
Thiobarbituric Acid

Further, either test yields its characteristic pigments for fat oxidation when applied to malonic dialdehyde *(5, 6)*.

As a result of the early work by Powick *(8)*, it has been stated widely in the literature that the compound from oxidized fats responsible for a positive Kreis test is epihydrin aldehyde.[b] Powick concluded that the compound did not exist as such in oxidized fat but that it was formed when the fat came in contact with concentrated HCl in the test. Doubt regarding the role of epihydrin aldehyde in the Kreis test was raised when a volatile component from oxidized milk fat, positive to both Kreis and TBA tests, was observed to have properties in keeping with those predicted for malonic dialedhyde but not for epihydrin aldehyde *(6)*.



Epihydrin Aldehyde
(Glycidaldehyde)



Malonic Dialdehyde
(enol form)

This problem seems to have been clarified considerably by two recent investigations. Payne *(7)* synthesized pure epihydrin aldehyde and observed that it is negative in the Kreis test. On the other hand Sinnhuber *et al (9)* have shown rather conclusively that the TBA pigments from malonic dialdehyde and oxidized salmon oil are identical and involve condensation of a three carbon unit with two moles of thiobarbituric acid. Their excellent paper also reviews a fair portion of the now substantial literature on the TBA test from its inception as a method of measuring aerobic metabolites in

[a] Authorized for publication on August 10, 1959 as Paper No. 2393 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

[b] More recently referred to as glycidaldehyde *(7)*, which compound was supplied to the author for experimental use through courtesy of the Shell Development Co., Emeryville, Cal.

tissues *(4)* to more recent and broad application in the fat stability problem of foods.

Payne's observation that epihydrin aldehyde is negative in the Kreis test was confirmed at this laboratory. It was noted also that at a level of 10 mcl the compound gives no evidence of a red pigment in the TBA test. The same quantity of malonic dialdehyde (as its diethyl acetal) gives red color in the TBA test to such an intensity that the pigment partially precipitates. However, epihydrin aldehyde did yield a golden yellow color in the test, the absorption spectrum for which is presented in Figure 1. The absorption

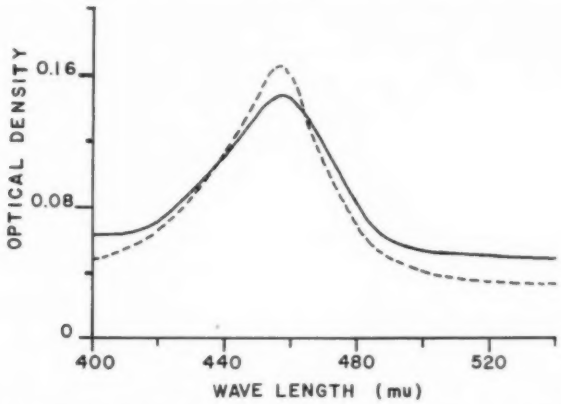


Figure 1. Response of epihydrin aldehyde (————) and glyceraldehyde (- - - - - -) in the TBA test.

maximum (456 mmc) for this yellow pigment is similar to that noted by Wilbur *et al (11)* for fructose. According to Payne, aqueous solutions of epihydrin aldehyde yield glyceraldehyde on standing in the presence of an acid catalyst. Since such a conversion might occur during performance of the TBA test, glyceraldehyde was evaluated at a level of 10 mg. It yielded the same golden yellow color as did epihydrin aldehyde. Moreover, the two compounds gave pigments having very similar wavelength maxima and optical densities (Fig. 1). Tested at the same level, dihydroxy-acetone yielded a negligible degree of yellow color. The TBA test was conducted as described by Wilbur *et al (11)* and the results in Figure 1 were observed to be reproducible.

Many systems submitted to the TBA test have yielded an appreciable degree of absorption in the 440-460 mmc (yellow) region *(1, 2, 3, 10, 11)*. Thus, although epihydrin aldehyde does not give the characteristic red color, its possible significance in the test cannot yet be completely discounted. In fact, its response in producing a yellow color raises the possibility of a TBA pigment from lipids and sugars having common origin in glyceraldehyde. An alternative explanation is that the pigments from epihydrin aldehyde and glyceraldehyde are not identical although spectrally coincident. This latter alternative cannot be easily dismissed in view of the large number of compounds which give yellow reactions in the test.

(After this note was in press new information of much interest became available, and it is summarized in the footnote below.[c])

In its capacity to measure various changes produced in food and biological systems by heat, light, air, ionizing radiations, and trace metals; the TBA test is truly remarkable and deserving of continued study.

[c] In the development of a colorimetric method for estimating 2-deoxy sugars, based on the malonic dialdehyde-TBA reaction, Waravdekar and Saslaw (*J. Biol. Chem.*, **234**, 1945, 1959.) observed that malonic dialdehyde and epihydrin aldehyde diethyl acetal yield indistinguishable products with TBA as judged by examination of visible spectra. Further study of this matter by Saslaw and the writer has established that the pink color arising from the acetal in the test was, in fact, due to an impurity by virtue of the low extinction for the pink pigment from one sample of the acetal, the complete absence of pink color from a second sample and the essentially identical infrared spectra exhibited by the two acetal samples prepared by two different methods.

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